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MOLECULAR PATHOGENESIS OF *LEGIONELLA LONGBEACHAE*

By

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B.Sc. University of Ghana, Legon, 1998

A Dissertation

Submitted to the Faculty of the Graduate School of the University of Louisville in
Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Microbiology and Immunology
University of Louisville
Louisville, Kentucky

May 2006

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DEDICATION

This dissertation is dedicated to my parents

The late Mr. Akwasi Asare

And

Mrs. Adwoah Ampomah

Without whose toil I would never have reached where I am today.

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I would like to thank my mentor, Dr. Yousef Abu Kwaik, without whose guidance and patience this work would not have seen the light of day. I would also like to thank the other committee members for their comments and criticism. Special thanks to the members of my lab; both past and present including Alaeddin Abu Zant, Maelle Molmeret, Snake Jones, Marina Santic, Maria T. Garcia Esteban, Fabian Habyarimana, Souhaila M. Al Khodor, and all the others for being there for me when I needed their help. Also many thanks to members of my family: my mom Adwoah Ampomah, my sisters Regina Asare, Agnes Asare, Georgina Asare, my brother Isaac Asare, and my cousin Charles Adumatta and his wife Abena Afrakomah. Special thanks to Dr. Bruce, Maley and Mary Gail Engle in the imaging facility of University of Kentucky for their help with the confocal imaging above and beyond the call of duty. I cannot forget Chris Price who helped me with my Real Time PCR analysis.

ABSTRACT

MOLECULAR PATHOGENESIS OF *LEGIONELLA LONGBEACHAE*

Rexford Asare

May 13, 2006

The ability of *L. pneumophila* to cause disease depends on its replication in alveolar macrophages. Infectivity and the expression of various virulence traits is triggered at post exponential growth phase. We show that unlike quiescent macrophages, *L. longbeachae* does not replicate in IFN- γ activated macrophages and its intracellular replication is independent of the growth phase of the inocula. *L. pneumophila* replicates in a phagosome that excludes early and late endocytic markers, and is surrounded by the rough endoplasmic reticulum (RER). In contrast, the *L. longbeachae* phagosome co-localizes with early endosomal marker EEA1, late endosomal markers LAMP-2 and M6PR, and ER marker KDEL, but excludes vacuolar ATPase (vATPase), and lysosomal markers cathepsin D, and Texas red ovalbumin. During late stages of infection, *L. longbeachae*, like *L. pneumophila* escapes into the cytoplasm, prior to lysis of the macrophage. Despite the different trafficking of *L. longbeachae* and *L. pneumophila*, both can replicate in communal phagosomes harboring both species. The *L. pneumophila* *dotA* mutant is rescued for intracellular replication if it co-inhibits the same phagosome with *L. longbeachae*. Most inbred mouse strains are resistant to infection by *L. pneumophila*, which is associated with polymorphism in the *naip5* allele. Most human homologues of Naip proteins inhibit caspase-3 and caspase-7 activity. *L. pneumophila*

activates caspase-3 which is necessary for arrest of phagosome biogenesis. We show that genetic susceptibility of several inbred mice strains to *L. longbeachae* is independent of allelic polymorphism of *naip5*, and infection of cultured murine macrophages by *L. longbeachae* is independent of polymorphism in the *naip5* allele. Real time PCR on permissive and resistant mouse macrophages shows that both *L. longbeachae* and *L. pneumophila* trigger similar level of *naip5* expression but the level of *naip5* is higher in macrophages resistant to *L. pneumophila*. Unlike *L. pneumophila*, *L. longbeachae* induces low level of caspase-3 activation and apoptosis during late stages of infection of macrophages *in vitro* or in the lungs of infected mice. Our data indicate a unique trafficking of *L. longbeachae* compared to other intracellular pathogens, and divergence in the intracellular life style of *L. longbeachae* from that of *L. pneumophila*.

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INTRODUCTION

I. The History and Epidemiology and Etiology of Legionnaires' disease

During the Legion Convention in Pennsylvania in the summer of 1976, a severe pneumonia outbreak occurred that affected 182 conference attendees resulting in 29 fatalities (Fraser, Tsai et al. 1977; McDade, Shepard et al. 1977). Later, the etiologic agent was isolated, and determined to be a facultative intracellular gram-negative bacterium (McDade, Shepard et al. 1977). The bacterium was designated *Legionella pneumophila* and the strain was named Philadelphia 1 (Brenner, Steigerwalt et al. 1979). Based on DNA comparisons, it was found that bacterial responsible for Legionnaires' disease were not related to any previously described species. Currently there are ~50 species designated *Legionella*, with 70 serogroups and approximately half can cause disease in humans (Benin, Benson et al. 2002).

Clinically, Legionnaires' Disease is characterized primarily by an acute pneumonia diarrhea, dry cough, malaise, high fever with relative bradycardia and pleuritic pain (Kirby, Snyder et al. 1978; Kirby, Snyder et al. 1980; Blackmon, Chandler et al. 1981). Clinical symptoms are however indistinguishable from pneumonia caused by many other etiologic agents (Edelstein and Meyer 1984; Sopena, Sabria-Leal et al. 1998). In addition to the typical Legionnaires' disease characterized by pneumonia, a mild form of the disease known as Pontiac fever which is a febrile self limited illness can also occur

(McDade, Shepard et al. 1977; Glick, Gregg et al. 1978; Rowbotham 1980; Kaufmann, McDade et al. 1981). The two diseases together are referred to as Legionellosis (Blackmon, Chandler et al. 1981).

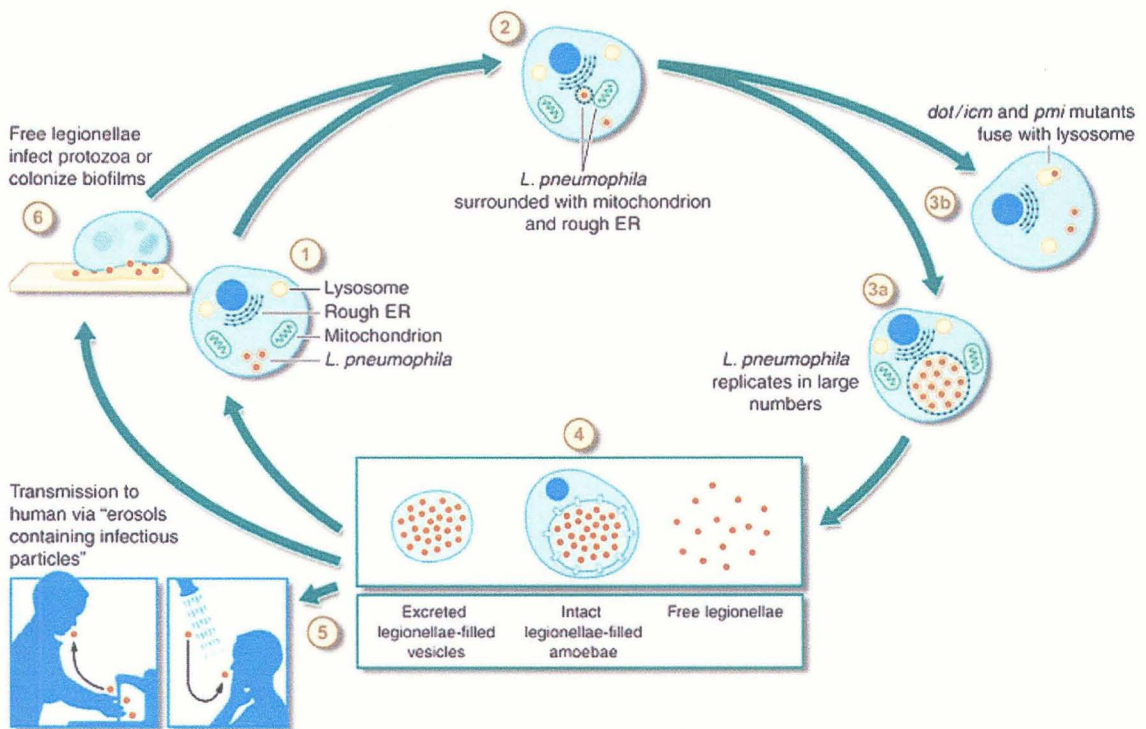
Legionella pneumophila accounts for 90% of all cases of Legionnaires' disease in the United States (Benin, Benson et al. 2002). Interestingly, in contrast the predominant species responsible for Legionnaires' disease in Western Australia is *L. longbeachae* (Doyle, Steele et al. 1998). *L. longbeachae* was first isolated from patient in Longbeach, California in 1981 and described as a new species of *Legionella* (McKinney, Porschen et al. 1981). It is now recognized that Legionnaires' disease occurs sporadically or in outbreaks in both community and nosocomial settings. It is estimated that ~18,000 people are hospitalized annually in the United States as a result of pneumonia caused by *Legionella*. It is believed that this number may be an underestimation due to the difficulty in isolating these bacteria and the similarity of the clinical symptoms of to those of other pneumonias. The elderly, smokers, and immunocompromised individuals are at higher risk of contracting Legionnaires' disease (England and Fraser 1981; Doebbeling and Wenzel 1987).

II. Ecology of *Legionella pneumophila* and *Legionella longbeachae*

After the initial 1976 outbreak, a link was quickly established between natural and man-made water sources and Legionnaires' disease (Fliermans, Cherry et al. 1979; Morris, Patton et al. 1979; Cordes, Fraser et al. 1980; Dondero, Rendtorff et al. 1980; Mahoney, Hoge et al. 1992). It is now established that *L. pneumophila* is ubiquitous in aquatic environments, and that infection occurs through inhalation of aerosols from the

contaminated water (Zimmerman 1979; Berendt 1980; Berendt, Young et al. 1980; Baskerville, Fitzgeorge et al. 1981; Arnow, Chou et al. 1982; Davis, Winn et al. 1982; Meenhorst, Reingold et al. 1983; Zuravleff, Yu et al. 1983).

Life Cycle of *L. pneumophila* within the Aquatic Environment



Amoeba in water serve as a reservoir for the amplification and dissemination of *L. pneumophila*, and are considered the natural host of these bacteria (Rowbotham 1980; Rowbotham 1981; Anand, Skinner et al. 1983; Henke and Seidel 1986; Rowbotham 1986; Fields, Sanden et al. 1989; Fields 1993). Moreover, growth of *L. pneumophila* in amoeba enhances its virulence (Cirillo, Tompkins et al. 1994; Cirillo, Cirillo et al. 1999). It has been shown that *L. longbeachae* in contrast, is unable to replicate in *Acanthamoebae castillanii* and *Hartmannella vermiformis* but shows moderate growth in the protozoan *Tetrahymena pyriformis* (Wadowsky, Wilson et al. 1991; Steele and

McLennan 1996; Neumeister, Schoniger et al. 1997). The ability of *L. pneumophila* to replicate within protozoa may explain, “the infective dose paradox” (O'Brein and Bhopal 1993). This paradox is based on the fact that aerosol droplets contain few free living *L. pneumophila* and likely insufficient to cause disease. However, aerosolized amoebae infected with *L. pneumophila* may contain several million bacteria sufficient to cause disease (O'Brein and Bhopal 1993). Man-made devices that generate sufficiently large aerosolized water droplets include cooling towers, showerheads, evaporative condenser, grocery store mist machines, and whirlpools, and these have all have been associated with Legionnaires' disease outbreaks. It is believed that the appearance of Legionnaires' disease is closely linked to these water handling devices (Morris, Patton et al. 1979; Cordes, Fraser et al. 1980; Dondero, Rendtorff et al. 1980; Breiman, Fields et al. 1990; Mahoney, Hoge et al. 1992).

The specific nature of the aerosolized infectious *Legionella*-containing particle is not known, but may contain free living bacteria, amoebae containing replicating bacteria, or excreted vesicles from amoebae that contain the bacteria (Rowbotham 1980; O'Brein and Bhopal 1993; Abu Kwaik, Gao et al. 1998). Unlike *L. pneumophila*, which mostly inhabits aquatic environments, *L. longbeachae* occurs predominantly in moist potting soil (Steele, Lanser et al. 1990; Koide, Saito et al. 1999; 2000). Thus, whereas *L. pneumophila* infection occurs through the inhalation of contaminated aerosol from water-handling devices, *L. longbeachae* infection occurs through inhalation of aerosol associated with contaminated potting soil.

III. The Intracellular Life Cycle of *Legionella* within its Host Cells

A. The life cycle of Legionella within its protozoan host cells

Following the attachment of *L. pneumophila* to its protozoan host, bacterial uptake occurs through conventional or coiling phagocytosis (Abu Kwaik 1996; Bozue and Johnson 1996). By 1 h to 2.5 h after entry, *L. pneumophila* is localized within a membrane-bound vacuole, which becomes surrounded by mitochondria and smooth vesicles. The phagosome is transformed into a ribosome studded multilayer membrane that is derived from the rough endoplasmic reticulum (RER) and shows co-localization with endoplasmic reticulum specific proteins (Abu Kwaik 1996). The *L. pneumophila*-containing phagosome (LCP) is inhibited from fusion with the lysosomes (Bozue and Johnson 1996). By 4 hours post infection, *L. pneumophila* begins to replicate within the RER derived vacuole suggesting that the early period of infection may be necessary for adaptation of *L. pneumophila* to its host cell (Abu Kwaik 1996; Bozue and Johnson 1996). The trafficking of *L. longbeachae* in protozoa has not been carried described. However Neumeister et al has shown that whereas *L. pneumophila* shows initial replication in *Acanthamoebae castellanii*, *L. longbeachae* does not replicate in this amoebae, indicating that there is a difference in the trafficking of the two species within this amoebae (Neumeister, Schoniger et al. 1997).

L. pneumophila replicates within the host cell until between 24 and 72 hours at which time the protozoan host is killed by a necrotic mechanism followed by release of the intracellular bacteria. The killing of the host cell occurs by a pore-formation mediated process (Gao and Abu Kwaik 2000). Furthermore, the killing of *A. polyphaga* by *L. pneumophila* has been described to be a mono-phasic event in which necrosis is the only

mode of bacterial induced host-cell killing (Gao and Abu Kwaik 2000). Interestingly, this event appears to depend on the intracellular growth phase of *L. pneumophila* and is activated upon entry into the terminal stage of intracellular replication (Gao and Abu Kwaik 2000).

B. The life cycle of Legionella within mammalian cells

i. Attachment and Entry into macrophages

L. pneumophila has been observed to replicate primarily in alveolar macrophages in the lung tissue sections from infected humans, or animal models of Legionnaires' disease. Moreover mutants which are not able to replicate in macrophages cannot cause infection in animal models of the disease (Chandler, Cole et al. 1979; Rodgers 1979; Winn and Myerowitz 1981; Baskerville, Dowsett et al. 1983; Baskerville, Fitzgeorge et al. 1983). In addition to the alveolar macrophages, replication of *L. pneumophila* in polymorphonuclear cells and epithelial cells has been reported (Baskerville, Dowsett et al. 1983; Mody, Paine et al. 1993).

Similar to the entry of *L. pneumophila* into protozoa, entry into macrophages has been shown to occur through both conventional and coiling phagocytosis (Horwitz and Silverstein 1981; Horwitz 1984). Entry of *L. pneumophila* to macrophages has been shown to be enhanced by pre-opsonization of the bacteria by specific *L. pneumophila* antibodies (Horwitz and Silverstein 1981; Nash, Libby et al. 1984; Husmann and Johnson 1992). This is further augmented by the presence of complement since normal serum enhances the uptake of *L. pneumophila* by macrophages in the presence of specific

antibodies (Horwitz and Silverstein 1981; Payne and Horwitz 1987; Bellinger-Kawahara and Horwitz 1990; Husmann and Johnson 1992). There is however a conflicting results on the role of complement receptor in the uptake of *L. pneumophila*. Whereas Horwitz et al. (Payne and Horwitz 1987) found that complement alone could enhance uptake, Johnson et al. (Husmann and Johnson 1992) found that complement alone could not enhance uptake. Complement-mediated uptake is thought to be a safe route of bacterial entry since engagement of the complement receptors does not induce an oxidative burst within macrophage (Wright and Silverstein 1983; Yamamoto and Johnston 1984). In contrast, uptake through the immunoglobulin receptor (FcR) induces the oxidative burst and phagosome-lysosome fusion (Wright and Silverstein 1983; Yamamoto and Johnston 1984). However, in the presence of both antibody and complement, only 50% of *L. pneumophila* are killed by macrophages (Horwitz and Silverstein 1981).

Potential bacterial ligands for binding to mammalian cell receptors include the major outer membrane protein (MOMP) and the type IV pili of *L. pneumophila* (Bellinger-Kawahara and Horwitz 1990; Stone and Abu Kwaik 1998). MOMP has been shown to be covalently modified by complement components (Bellinger-Kawahara and Horwitz 1990). Mutants that do not express the type IV pili exhibit reduced attachment to both macrophages and epithelial cells (Stone and Abu Kwaik 1998). A surface exposed heat-shock protein (Hsp60) has been shown to mediate attachment of *L. pneumophila* to HeLa epithelial cells (Garduno, Faulkner et al. 1998; Garduno, Garduno et al. 1998). Unlike *L. pneumophila*, uptake of *L. longbeachae* has not been studied.

ii. Intracellular fate of *L. pneumophila* with macrophages

In general, endocytosis of receptor-ligand complexes occurs through clathrin-coated vesicles (Sahagian and Steer 1985). Following endocytosis, the clathrin coat is rapidly lost to allow fusion of these vesicles with early endosomes (EE) or sorting endosomes (Mellman 1996). The early or sorting endosome compartment is slightly acidic (pH of 6.0-6.8), which allows the receptor-ligand complexes to dissociate at this point so that recycling of molecules such as the transferrin receptor (TfR) back to the cell surface can occur (Mellman 1996). Early endosomes associate with small Rab GTPases such as Rab4, Rab5 and Rab11. The GTPases regulate fusion of the endosomes to other endocytic vesicles. There is rapid maturation of early endosomes into late endosomes (LE), a process that is manifested by dynamic changes in the composition of the endosome. The early endosomal GTPases are excluded from the late endosomes and are replaced by late endosomal GTPases including Rab7 and Rab9 (Desjardins, Huber et al. 1994; Mellman 1996). In addition, the LE is enriched in ATPase pump and several highly glycosylated and conserved proteins such as lysosomal membrane associated proteins (LAMP-1 and -2), and two pH-sensitive mannose-6-phosphate receptors (M6PRs) (Mellman 1996). Subsequently, the late endosome fuses to lysosomes where internalized particles or macromolecules are digested by lysosomal enzymes. Lysosomes contain lysosomal glycoproteins and hydrolases but are devoid of M6PRs. Similar to endocytosed particles; inert particles that are engulfed by phagocytic cells follow the same route and are digested in a phagolysosome.

After phagocytosis, *L. pneumophila* localizes to a unique phagosome that is isolated from the endocytic pathway (Horwitz 1983; Horwitz 1983; Horwitz and Maxfield 1984). The LCP excludes endocytic markers, including the lysosomal

associated membrane glycoproteins, Rab5, LAMP-1 and LAMP-2 as well as the lysosomal acid protease cathepsin D (Clemens and Horwitz 1995; Clemens, Lee et al. 2000; Clemens, Lee et al. 2000). The *L. pneumophila* phagosome is only mildly acidified (pH of 6.2-6.3) resembling the pH of an early endosome (Horwitz and Maxfield 1984; Mellman 1996). At the ultra structural level the characteristics of the *L. pneumophila* phagosome within mammalian and protozoan cells are similar (Abu Kwaik 1996).

Within 5 minutes of entry, the majority of *L. pneumophila* phagosomes are devoid of rab5, rab7 and LAMP-1 while attenuated mutants of *L. pneumophila* reside within a LAMP-1 positive compartment (Sinai and Joiner 1997; Roy, Berger et al. 1998). Thus, within minutes of entry the *L. pneumophila* phagosome is inhibited from maturation along the classical endosomal/lysosomal pathway. In addition, at 3hs hours post-infection of macrophages, the *L. pneumophila* phagosome is also devoid of the late endosomal/lysosomal markers LAMP-1, LAMP-2, Cathepsin-D, and CD-63 (Clemens and Horwitz 1995)

In addition to the isolation from the endocytic pathway, the LCP recruits the endoplasmic reticulum (Horwitz 1983; Swanson and Isberg 1995). Using electron microscopy, Tilney et al (2001) showed that the LCP becomes surrounded with smooth vesicles from the ER which zippers around the phagosome within 15 mins of entry into macrophages (Tilney, Harb et al. 2001). By 6 h post infection, there is fusion between the vesicles and the phagosome and ribosomes begin to attach to the LCP membrane. Initially, the LCP membrane resembles the plasma membrane with a thickness of $\sim 70\text{\AA}$ but converts quickly to a 60\AA thick membrane (Tilney, Harb et al. 2001). Recruitment of the ER occurs by intercepting vesicular trafficking at the exocytic exit site (Kagan and

Roy 2002). To achieve the recruitment of the early secretory vesicles to create its replicative organelle, *L. pneumophila* subverts Rab1 and Sec22b which are necessary for trafficking between the ER and the golgi (Derre and Isberg 2004; Kagan, Stein et al. 2004). Similar to *L. pneumophila*, the *L. longbeachae* containing phagosome becomes surrounded with the ER (Gerhardt, Walz et al. 2000; Doyle, Cianciotto et al. 2001). The evasion of endosytic fusion and the recruitment of the ER are mediated by the *dot/icm* type IV secretion system. *L. pneumophila* begins to replicate by 4 hours post infection and eventually kills its host cell and is released into the extracellular environment through the actions of the *Legionella* pore-forming activity (Abu Kwaik 1998; Alli, Gao et al. 2000).

iii. Interaction between L. pneumophila and autophagosomes

Autophagy is a mechanism by which eukaryotic cells capture unwanted cytoplasmic components and damaged organelles for delivery to the lysosomes (Dunn 1990; Seglen, Gordon et al. 1990). A hallmark of autophagy is the sequestration of the cytoplasmic components into a double-membrane vacuole known as the autophagosome (Dunn 1990; Seglen and Bohley 1992). As the autophagosome matures, it acquires late endosomal/lysosomal markers such as LAMP-2 (Dunn 1990; Kopitz, Kisen et al. 1990). Interestingly, it has been shown that *L. pneumophila* modulate maturation of its phagosome through autophagy and this process is Dot/Icm-dependent (Swanson and Isberg 1995; Amer and Swanson 2005). Although there is a general presumption that trafficking of *Legionella* species in mammalian cells is likely to be similar, trafficking of

L. longbeachae has not been reported, and it is possible that *Legionella* species differ in their trafficking.

iv. Egress of L. pneumophila from protozoa and Mammalian cells

Although the initial infection appear similar between the intracellular infection of mammalian and protozoan cells, there is a marked difference in the killing mechanisms (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Alli, Gao et al. 2000; Gao and Abu Kwaik 2000; Harb, Gao et al. 2000). *L. pneumophila* utilizes two mechanisms to kill macrophages (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Alli, Gao et al. 2000), in contrast to a single mechanism for protozoa (Gao and Abu Kwaik 2000; Harb, Gao et al. 2000). At late stages of infection of mammalian cells, there is induction of caspase-3 mediated apoptosis (Abu-Zant, Santic et al. 2005) followed by a necrotic cell death that is mediated by the *L. pneumophila* pore-forming activity (Alli, Gao et al. 2000). The pore-forming activity leads to lysis of the host cell and release of intracellular bacteria since mutants which lack the pore-forming activity replicates normally but are not able to exit the cell at the end of the replicative cycle (Alli, Gao et al. 2000). In contrast, egress from amoeba is mediated by the pore-forming activity and *L. pneumophila* does not trigger apoptosis in amoeba (Gao and Abu Kwaik 2000).

IV. Activated Macrophages inhibits growth of *L. pneumophila*

The containment and clearance of many intracellular pathogens requires the activation of macrophages by IFN- γ (Neild and Roy 2004). Several lines of evidence indicate that IFN- γ plays an important role in the elimination of *L. pneumophila* from the

lungs of infected animals. A study using antibody depletion showed that the clearance of *L. pneumophila* from the lung of infected A/J mouse is dependent on host production of IFN- γ (Brieland, Freeman et al. 1994). In addition, it has been shown that IFN- γ knockout mice is unable to clear *L. pneumophila* infections as effectively as wild type mice (Heath, Chrisp et al. 1996). Within 6 h post infection, IFN- γ is detected in the serum of mice infected intratracheally with *L. pneumophila*, and bacterial clearance occurs as early as 72h after infection (Brieland, Freeman et al. 1994). It has been shown that the first source of IFN- γ production after infection of the host cell with *L. pneumophila* is likely to be natural killer (NK) cells (Blanchard, Friedman et al. 1988; Deng, Tateda et al. 2001);

It is known that *L. pneumophila* can grow exponentially within human monocytes and macrophages derived from the A/J strain of mouse (Horwitz and Silverstein 1980; Yamamoto, Klein et al. 1988). However upon activation with IFN- γ , these cells no longer support the growth of *L. pneumophila* (Horwitz and Silverstein 1981; Klein, Yamamoto et al. 1991). Growth of *L. pneumophila* in monocytes and alveolar macrophages is restricted in a dose dependent manner upon activation with IFN- γ (Bhardwaj, Nash et al. 1986; Nash, Libby et al. 1988; Santic, Molmeret et al. 2005). This growth restriction is abrogated by the addition of anti-IFN- γ antibodies (Bhardwaj, Nash et al. 1986). Interestingly, while human monocytes treated with low amounts of IFN- γ restrict the growth of *L. pneumophila*, bacteria do not appear to be killed inside these cells.

It was determined that the restriction of *L. pneumophila* growth in IFN- γ -activated macrophages is in part due to sequestration of intracellular iron as a result of downregulation of transferrin receptor (Byrd and Horwitz 1989; Byrd and Horwitz

1991). Interestingly the *L. pneumophila* in activated human monocyte-derived macrophages and A/J mouse bone marrow-derived macrophages fail to remodel the biogenesis of its phagosome. The phagosome shows co-localization with the LAMP-2, Cathepsin D and Texas red ovalbumin markers (Santic, Molmeret et al. 2005). It will be interesting to study the relationship between the downregulation of the transferring receptor and maturation of the *L. pneumophila* containing phagosome. One of the pathways that are up regulated in IFN- γ activated infected macrophages is the production of nitric oxide (NO). Although NO has been found to play an important role in the immune response of A/J mice against *L. pneumophila*, the effect appears to be indirect (Neild and Roy 2004).

V. Growth Phase-Dependent Virulence of *Legionella*.

Several virulence phenotypes of *L. pneumophila* have been linked to the bacterial growth phase in vitro (Byrne and Swanson 1998) and in vivo (Alli, Gao et al. 2000; Garduno, Garduno et al. 2002). During the terminal stage of intracellular replication, *L. pneumophila* differentiate to a morphological distinct phenotype referred to as mature intracellular form (MIF). The MIF share several virulence properties with in vitro stationary phase. While in vitro grown post-exponential *L. pneumophila* can replicate intracellularly, exponentially-grown *L. pneumophila* is completely defective in subsequent intracellular replication (Byrne and Swanson 1998). In addition to its ability to replicate intracellularly, post exponential phase *L. pneumophila* exhibit other virulence-related phenotypes related to motility, sodium chloride sensitivity, cytotoxicity, resistance to osmotic lysis, and evasion of phagosome-lysosome fusion (Byrne and

Swanson 1998). Similarly, Alli *et al.* have shown that pore formation is growth phase-regulated both *in vitro* and *in vivo* (Alli, Gao et al. 2000).

These phenotypic changes occur in response to nutrient starvation (Hammer and Swanson 1999). In particular, depletion of five amino acids (serine, tyrosine, asparagine, proline, and threonine) appears to be crucial for the expression of the virulence traits in *L. pneumophila* (Hammer and Swanson 1999). Upstream regulators for the expression of the virulent traits include RelA, a ppGpp synthetase that is activated in the presence of non-charged tRNA, and the stationary phase sigma factor RpoS (Hammer and Swanson 1999; Zusman, Gal-Mor et al. 2002). Hammer et al. have shown that when exponentially growing bacteria are incubated in broth of post-exponential bacteria that is depleted of nutrients, the bacteria convert from the replicative phase to the virulent phase while RelA mutants fail to convert (Hammer and Swanson 1999). It is believed that ppGpp acts as an alarmone that activates the two-component regulator system, LetA/S, and this enables *L. pneumophila* to express its full arsenal of virulence properties at the post exponential phase (Hammer, Tateda et al. 2002; Molofsky and Swanson 2004).

In contrast to broth grown bacteria, *L. pneumophila* growing intracellularly is capable of converting from the non virulent replicative form to a virulent mature intracellular form (MIF) without the LetA/S two-component system (Hammer, Tateda et al. 2002). Thus, it is possible that there are unknown environmental signals that trigger activation of the virulence properties inside cells. It has been proposed that *L. pneumophila* represses virulence traits required for invasion during intracellular replication in a nutrient-rich intracellular environment. However, upon depletion of nutrients in spent host cells, the virulence traits are activated to trigger bacterial release

and allow for secondary infections (Abu Kwaik 1998; Molofsky and Swanson 2004). Since this regulation of virulence phenotypes has only been shown *in-vitro* it is impossible to eliminate other possible regulation of virulence traits such as quorum sensing, a system utilized in numerous other pathogens. It is likely that *L. pneumophila* possess several effector molecules and mechanisms that are involved in mediating this dramatic phenotypic transformation at the post exponential phase. Growth phase-dependent expression of virulence factors in other *Legionella* species has not been studied.

VI. Genes Required for Intracellular Replication of *Legionella pneumophila*

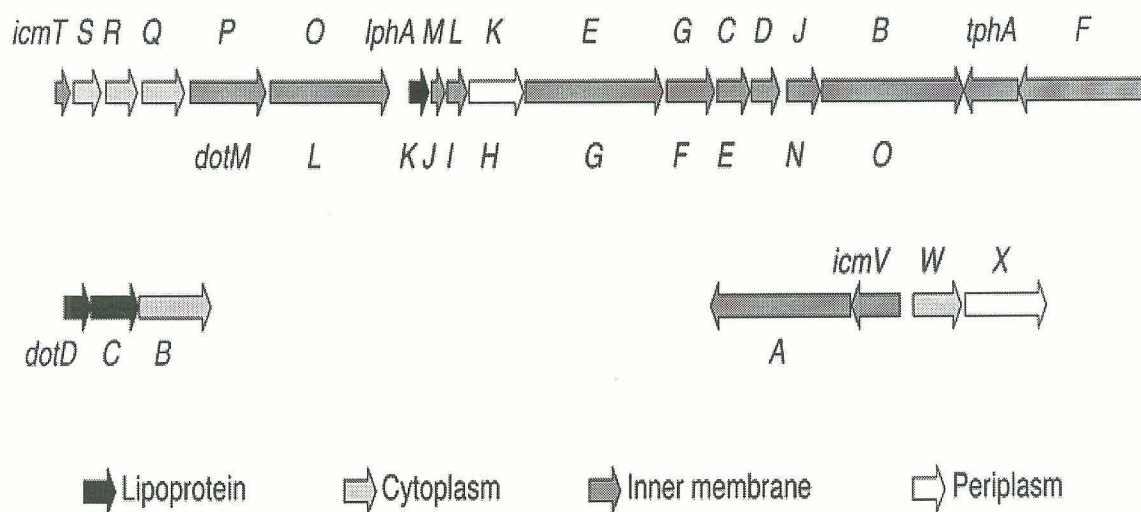
A. The *dot/icm* Type IV secretion genes

Bacterial pathogens have evolved a variety of conserved secretion systems to deliver effector proteins into their host to alter cell biology and host immunity. The alteration in the host cell biology and immunity allow the bacteria to survive and replicate within the host. Two of these secretion systems are evolutionarily similar to bacterial organelles. These are Type III secretion system which evolved from the flagellum assembly apparatus and the Type IV secretion system that evolved from the conjugation pilus machinery (Galan and Collmer 1999; Christie and Vogel 2000). The effector molecules secreted by the Type III secretion system possess a variety of functions that affect host cell biology (Galan and Collmer 1999). The Type IV secretion system of a number of bacterial pathogens has been shown to export effector molecules that interfere with cellular signaling within the host cell (Cascales and Christie 2003). *Bordetella pertussis* uses the Type IV secretion system to export the pertussis toxin which has ADP-

ribosylating activity that ribosylates the α -subunits of G protein thus interfering with intracellular signaling (Burns 1999; Christie and Vogel 2000).

The *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication) type IV secretion system is encoded by the same set of genes of *L. pneumophila*, that were independently identified by two different laboratories (Table 1). The first *dot/icm* was identified during the characterization of a spontaneous mutant of *L. pneumophila* (mutant 25D) generated by serial passage on Muller-Hinton media (Horwitz 1987). It was found that this mutant is defective in intracellular multiplication, recruitment of the RER, and inhibition of phagosome-lysosome fusion, and is resistant to sodium chloride (Horwitz 1987; Marra, Blander et al. 1992; Sadosky, Wiater et al. 1993). A *L. pneumophila* chromosomal fragment containing 4 open reading frames (*icmWXYZ*) complemented the defective phenotype of 25D (Marra, Blander et al. 1992; Sadosky, Wiater et al. 1993; Brand, Sadosky et al. 1994). The *icmXYZ* region was later determined to have one genes; *icmX* (Matthews and Roy 2000). Based on the initial characterization, transposon mutagenesis of the *L. pneumophila* chromosome was performed and 55 mutants were isolated that are defective for intracellular survival in macrophages (Sadosky, Wiater et al. 1993; Segal and Shuman 1999). Most of those mutations mapped to the *icmWXYZ* operon or adjacent genomic region (Sadosky, Wiater et al. 1993; Brand, Sadosky et al. 1994; Segal and Shuman 1997; Purcell and Shuman 1998; Segal, Purcell et al. 1998). Interestingly, most of the mutants were found to be resistant to sodium chloride when compared to the parental wild type strain of *L. pneumophila* (Sadosky, Wiater et al. 1993).

Initial characterization of the *dot* genes was performed using a thymine-free enrichment technique (Berger and Isberg 1993). Using this method, one mutant (*dotA*) that exhibited similar intracellular survival and lack of replication in the presence or absence of thymine was isolated (Berger and Isberg 1993). Phagosome containing the *dotA* mutant was found to fuse to lysosomes and was not surrounded by the RER (Berger and Isberg 1993). Subsequently, 26 spontaneous mutants of *L. pneumophila* were isolated based on their resistance to NaCl (Vogel, Roy et al. 1996). Chemically induced mutagenesis was also used to isolated 6 additional *dot* mutants (Andrews, Vogel et al. 1998). Genetic analysis and complementation of these mutants led to the identification of 15 *dot* genes organized in two unlinked regions on the *L. pneumophila* genome (Vogel, Roy et al. 1996; Vogel, Andrews et al. 1998). In all, a total of 25 *dot/icm* genes have been identified that are necessary for intracellular replication of *L. pneumophila* (Marra, Blander et al. 1992; Berger and Isberg 1993; Sadosky, Wiater et al. 1993; Brand, Sadosky et al. 1994; Vogel, Roy et al. 1996; Segal and Shuman 1997; Andrews, Vogel et al. 1998; Segal, Purcell et al. 1998; Vogel, Andrews et al. 1998).



Analysis of the predicted amino acid sequences of the *dot/icm* genes has revealed several characteristics about their possible functions and their sub cellular localization (Table 1). First, most of the *dot/icm* genes exhibit homologies to proteins involved in conjugal transfer of plasmid DNA. DotB is homologous to several nucleotide-binding proteins found in conjugative transfer systems including PilT, PulE, and ComG (Vogel, Roy et al. 1996). In addition, the predicted proteins encoded by *dotG* share considerable homology with conserved proteins TrbI and VirB10 of type-IV secretion systems (Vogel, Andrews et al. 1998). Second, the wild type *L. pneumophila* is able to transfer the IncQ plasmid, RSF1010, to another strain of *L. pneumophila* or to *E. coli* in contrast to *dot/icm* mutants, which are defective in the transfer of the IncQ plasmid (Segal, Purcell et al. 1998; Vogel, Andrews et al. 1998). Third, sequence analysis has revealed that many of the *dot/icm* genes encode integral membrane proteins indicative of their role as components of a protein transfer system (Roy and Isberg 1997; Segal and Shuman 1998). Fourth, four of the *dot/icm* genes appear to contain ATP/GTP-binding domains similar to proteins found in other Type IV secretion systems (Segal and Shuman 1998). All of these features support the idea that the *dot/icm* genes encode proteins involved in the assembly of a secretion apparatus. Interestingly, *dot/icm* mutants are defective in early events required for proper maturation of the LCP (Kirby, Vogel et al. 1998; Segal, Purcell et al. 1998; Vogel, Andrews et al. 1998).

Table. 1 The Dot/Icm Type IV secretion Loci and possible homologues

Protein	R64 (IncI)	Vir/IncP(RP4)
IcmT	TraK	

IcmS		
IcmR		
IcmQ		
IcmP/DotM		TrbA
IcmO/DotL	TraG	VirD4/TrbC
LphA,IcmN/DotK		
IcmM/DotJ		
IcmL/DotI	TraM	
IcmK/DotH		
IcmE/DotG	Tra0	VirB10/TrbI
IcmG/DotF	TraP	
IcmC/DotE	TraQ	
IcmD/DotP	TraR	
IcmJ/DotN	TraT	
IcmB/DotO	TraU	VirB4/TrbE
IcmF		
IcmH		
IcmV	TraX	
IcmX	TraW	
IcmW		

DotA	TraY	
DotB	TraJ	VirB11/TrbB
DotC	TraI	
DotD	TraH	

B. The Dot/Icm Type IV secretion substrates

Several protein substrates have been found to be translocated by the Dot/Icm type IV secretion system. These include RalF, LidA, SidA-SidH, and YlfA (Nagai and Roy 2001; Nagai, Kagan et al. 2002; Conover, Derre et al. 2003; Luo and Isberg 2004; Campodonico, Chesnel et al. 2005). Thus, the *dot/icm* genes are involved in the assembly of a type-IV-like secretion system that is involved in the transport of macromolecules required for proper trafficking of the *L. pneumophila* containing phagosome (Kirby, Vogel et al. 1998; Segal, Purcell et al. 1998; Vogel, Andrews et al. 1998). Functions of some of the Dot/Icm secretion substrates are known whereas others are yet to be determined. The RalF protein is translocated to the cytoplasmic face of the phagosomal membrane within the macrophage and is absent from the phagosome of *dot/icm* mutants (Nagai, Kagan et al. 2002). RalF acts as an exchange factor for ADP ribosylation factor-1 (ARF-1), a protein belonging to the ADP ribosylation factor family for the guanosine triphosphatases (GTPases), which act as key regulators of vesicular traffic from the ER and the Golgi apparatus (Nagai, Kagan et al. 2002). Other Dot/Icm secretion substrates are the substrate of Icm/Dot transporter (Sid) proteins (Luo and Isberg 2004). These include SidA-SidG. Whereas the SidA, SidD, SidF, and SidG proteins have no paralogues, the rest of the Sid proteins have between 1-4 paralogues with SidB having

one orthologue. One of the Sid proteins, SidC, is translocated to the cytoplasmic of the *L. pneumophila* containing phagosome (Luo and Isberg 2004). Whereas most of Dot/Icm exported substrates do not contribute individually to intracellular replication within macrophages, a mutation in a paralogue of SidE, SdeC produced a detectable defect in intracellular multiplication (Luo and Isberg 2004). LidA and YlfA are other Dot/Icm substrates, which have been shown to translocate to the cytoplasmic surface of the *L. pneumophila* containing phagosome (Conover, Derre et al. 2003; Campodonico, Chesnel et al. 2005). Both the LidA and YlfA have been found to interfere with the secretory pathway of the host cell although mutation in these genes did not result in any apparent phenotype (Campodonico, Chesnel et al. 2005; Derre and Isberg 2005).

L. longbeachae possess an intact type IV secretion system (Feldman and Segal 2004). It was shown by Feldman and Vogel that instead of *icmQ*, *L. longbeachae* has a *liga* gene, which complements for loss of *icmQ* from *L. pneumophila* (Feldman and Segal 2004). This indicates that *L. longbeachae* possesses a functional *dot/icm* secretion system.

VII Modulation of Apoptosis by *L. pneumophila*

A. Initiation of Apoptosis

Apoptosis is a form of programmed cell death in which a controlled sequence of events (or program) leads to the elimination of cells without causing inflammation in the surrounding tissue (Arends and Wyllie 1991; Cohen 1993; Savill, Fadok et al. 1993). Cells that are undergoing apoptosis display a characteristic morphological change that

includes membrane blebbing and nuclear condensation (Arends and Wyllie 1991). Cysteine proteases known as caspases play a central role in the execution of apoptosis within a cell (Cohen 1997). This is supported by the fact that animals that have been genetically altered to lack certain caspases show intense defects in apoptosis during development (Cohen 1997). There are two initiator caspases (caspase-8 and caspase-9) that mediate apoptosis leading to activation of two distinct signaling pathways designated as extrinsic and intrinsic pathways respectively (Strasser, O'Connor et al. 2000; Chen and Wang 2002; Shi 2002). Both pathways converge on the activation of the effector caspase, Caspase-3, which is responsible for the subsequent cellular and biochemical alterations, observed in apoptotic cells.

B. Effect of Rabaptin-5 cleavage by caspase-3 on endosomal fusion

Apoptotic cells exhibit severe disruption of intracellular trafficking leading to the formation of large vesicles as a result of inhibition of endocytic fusion (Cosulich, Horiuchi et al. 1997). This is as a result of the cleavage of the Rab5 effector protein, Rabaptin-5 by Caspase-3 leading to destabilization of the Rab5 complex (Cosulich, Horiuchi et al. 1997). When Caspase-3 cleaves Rabaptin-5 in *Xenopus* egg extracts the rate of vesicular fusion is decreased substantially and can only be restored by the addition of recombinant Rabaptin-5 (Cosulich, Horiuchi et al. 1997). Moreover, the addition of active Caspase-3 to extracts that have been previously depleted of all Caspase-3 by immunoprecipitation restores the cleavage of Rabaptin-5 and blocks endocytic fusion (Swanton, Bishop et al. 1999). Furthermore, inhibitors specific for Caspase-3 completely prevent the cleavage of Rabaptin-5 and restores endocytic fusion. Thus, the cleavage of

Rabaptin-5 by Caspase-3 is solely responsible for the inhibition of endocytic fusion (Cosulich, Horiuchi et al. 1997).

Caspase-3 cleaves Rabaptin-5 at the sequence DESD⁴³⁸ after the aspartate residue and at a secondary site HSLD³⁷⁹, both found within a loop region located between two large helices that account for the majority of the Rabaptin-5 protein (Swanton, Bishop et al. 1999). After caspase-3 cleaves Rabaptin-5, the two helices of the protein are separated and dissociated into the cytosol, as shown by immuno-precipitation experiments (Swanton, Bishop et al. 1999). Caspase-3-mediated cleavage of Rabaptin-5 would disrupt recruitment of downstream effectors of Rab5 on early endosome, thus, blocking endocytic fusion.

***C. Legionella pneumophila* induces caspase-3 activation during early stages of infection**

Both the extrinsic and the intrinsic pathway of apoptosis converge on activation of caspase-3, which is the executioner of apoptosis (Degterev, Boyce et al. 2003). Active caspase-3 directly cleaves more than 60 proteins, including PARP (Poly (ADP-Ribose) polymerase) and ICAD (Inhibitor of caspase activated DNase) (Cohen 1997; Enari, Sakahira et al. 1998) and caspases-6 also cleaves Lamin, and Fodrin (Cohen 1997) resulting in the induction of apoptosis.

L. pneumophila activates caspase-3 within U937 macrophages in a time- and dose dependent manner (Gao and Abu Kwaik 1999). The activation of caspase-3 by *L. pneumophila* is independent of both caspase-8 and caspase-9 (Molmeret, Zink et al. 2004). At low MOI (moi 5), caspase-3 is activated during the replicative phase but

induction of apoptosis does not occur until late stages of infection (Abu-Zant, Santic et al. 2005). The ability of *L. pneumophila* to induce apoptosis is inhibited by a specific inhibitor of Caspase-3, confirming that the activation of caspase-3 is directly responsible for the observed apoptosis (Gao and Abu Kwaik 1999). Activation of the apoptotic pathway is induced by extracellular *L. pneumophila* at high MOI, since inhibition of uptake by cytochlasin-D does not inhibit the induction of apoptosis within U937 cells (Gao and Abu Kwaik 1999). However preactivation of caspase-3 with Staurosporin or TNF- α inhibits *L. pneumophila* replication indicating that although caspase-3 activation is necessary for intracellular replication, apoptosis is delayed to allow the bacteria to replicate.

The activation of caspase-3 by *L. pneumophila* during early stages leads to the cleavage of the Rab5 effector Rabaptin-5 (Molmeret, Zink et al. 2004). Inhibition of caspase-3 activation inhibits replication of wild type *L. pneumophila* and causes the LCP to mature along the default endocytic pathway (Molmeret, Zink et al. 2004). Mutants in the Dot/Icm type IV secretion system are defective in the induction of caspase-3-mediated apoptosis and their phagosome matures into a phagolysosome (Zink, Pedersen et al. 2002). Thus, the Dot/Icm secretion system is required for the activation of caspase-3 and for evasion of endocytic fusion during early stages of infection by *L. pneumophila*. Although *L. longbeachae* has been shown to induce apoptosis through the caspase pathway, the role of individual caspases was not determined (Arakaki, Higa et al. 2002). Moreover the maximum level of apoptosis in *L. longbeachae* infected cells was shown to be ~30% in contrast to about ~80% for *L. pneumophila* (Gao and Abu Kwaik 1999; Arakaki, Higa et al. 2002).

VIII. Inbred strains of mice show differential susceptibility to *L. pneumophila* infection

Among inbred mice strains, A/J is the only inbred mouse strain susceptible to *L. pneumophila* infection. All the other strains are resistant (Hedlund, McGann et al. 1979; Yoshida and Mizuguchi 1986; Yamamoto, Klein et al. 1987; Yamamoto, Klein et al. 1988). In contrast, most inbred strains of mice are susceptible to infection by many other *Legionella* species (Miyamoto, Maruta et al. 1996; Izu, Yoshida et al. 1999). Studies in macrophages from F1, F1 backcross and F2 mice revealed that the unique permissiveness of A/J macrophages to *L. pneumophila* infection was recessive and linked to a difference in bacteriostatic activity (Yoshida, Goto et al. 1991). The unique genetic susceptibility has been attributed to a polymorphism in the neuronal apoptosis inhibitory protein (Naip)/baculoviral inhibitor of apoptosis repeat containing 1e (Birc1e) gene of the inhibitor of apoptosis (IAP) gene family (Yoshida, Goto et al. 1991; Beckers, Yoshida et al. 1995; Dietrich, Damron et al. 1995; Beckers, Ernst et al. 1997; Diez, Lee et al. 2003; Wright, Goodart et al. 2003). The *iap* family of genes are evolutionary conserved from viruses to humans, and some have been shown to possess anti-apoptotic activity (Clem and Miller 1994; Duckett, Nava et al. 1996; Uren, Pakusch et al. 1996). The anti-apoptotic activities are achieved through interaction of their baculovirus inhibitor of apoptosis repeat (BIR) domains with cellular caspases, including caspase 3 and 7 (Deveraux, Takahashi et al. 1997; Roy, Deveraux et al. 1997; Maier, Lahoua et al. 2002). Recently, a direct ATP-dependent inhibition of effector caspase-9 by Naip/birc1 BIR domains has been demonstrated *in vitro* (Davoodi, Lin et al. 2004; Fortier, Diez et al.

2005). In addition to the human homologues, a murine IAP homologue, mMIHA, has been shown to inhibit apoptosis mediated by interleukin 1 β converting enzyme (Uren, Pakusch et al. 1996). It is not known whether other murine homologues of the Naip proteins possess caspase inhibitory activity. In addition to the inhibition of caspases, Naip proteins achieve their anti-apoptotic effect through TAK1 dependent activation of the mitogen-activated protein (MAP) kinase, JNK1 (Sanna, da Silva Correia et al. 2002). Recently Naip proteins have been classified in the NACHT-LRR (NLR) family of proteins, which cytoplasmic proteins involved in intracellular recognition of microbial products (Chamaillard, Girardin et al. 2003; Fortier, Diez et al. 2005). Members of this family of proteins which include NODs, IPAFs and NALPs possess domains such as caspase-recruitment domain (CARD), BIR domains, and pyrin domain (PYD) respectively (Chamaillard, Girardin et al. 2003; Fortier, Diez et al. 2005). These domains are involved in the regulation of pro-apoptotic and pro-inflammatory signaling. IPAF and NALP subfamilies have been shown to activate the pro-inflammatory caspases including caspase-1 and caspase-5 to form inflammasome (Chamaillard, Girardin et al. 2003; Martinon and Tschopp 2004; Fortier, Diez et al. 2005)

At least 8 murine homologues of *naip* genes have been identified (Scharf, Damron et al. 1996; Yaraghi, Korneluk et al. 1998; Huang, Scharf et al. 1999; Growney, Scharf et al. 2000) and *naip5* has been identified as the gene responsible for the differential susceptibility of A/J mice to *L. pneumophila* infection (Growney and Dietrich 2000; Diez, Lee et al. 2003; Wright, Goodart et al. 2003). However, the role of the Naip5 protein in the activation of caspase-3 and apoptosis has not been determined. The family of Naip/birc1 proteins (at least 8) are expressed abundantly in macrophage-rich tissues in

mice and their collective expression is increased after phagocytosis by murine macrophages (Diez, Yaraghi et al. 2000). Whether Naip5 is one of the induced Naips is not known. The role of polymorphism of the *naip5* allele in the susceptibility of mice to *L. longbeachae* infection has not been studied.

Specific aims

Almost all studies of Legionnaires' disease have concentrated on *L. pneumophila*. It has been presumed that these studies could be extrapolated to other *Legionella* species. Preliminary work done on the intracellular trafficking of other species of *Legionella* such as *L. micdadei* indicate that trafficking of *L. pneumophila* is different from other *Legionella* species (Abu Kwaik, Venkataraman et al. 1998; Joshi and Swanson 1999; Gerhardt, Walz et al. 2000). *L. longbeachae* is the leading cause of Legionnaires' in Western Australia with sporadic cases recorded in Europe and the United States but very little is known about the pathogenesis of its infection. *L. longbeachae* replicate in macrophages from resistant inbred strains of mice compared to *L. pneumophila* (Yamamoto, Klein et al. 1988; Miyamoto, Maruta et al. 1996). As previously described, the differential susceptibility has been mapped to a polymorphism in the *naip5* allele, which belongs to the *naip/birc1* (IAP) family of genes (Yoshida, Goto et al. 1991; Beckers, Yoshida et al. 1995; Dietrich, Damron et al. 1995; Beckers, Ernst et al. 1997). Some members of this family of proteins inhibit caspase-3 and caspase-7 activity (Maier, Lahoua et al. 2002). My hypotheses are: (1) that intracellular trafficking of *L. longbeachae* is different from *L. pneumophila*, and (2) the differential susceptibility of *L.*

pneumophila to inbred strains of mice in contrast to *L. longbeachae* is associated with differences activation of caspase-3. To test these hypotheses, I intended to:

- Determine Intracellular trafficking of *L. longbeachae* in macrophages
- Characterize the intracellular infection of macrophages by *L. longbeachae* at the ultra-structural level.
- Determine if polymorphism in the *naip5* allele affects the susceptibility of mice to *L. longbeachae*.
- Characterize the expression of *naip5* in macrophages infected by *L. pneumophila* and *L. longbeachae*.

MATERIALS AND METHODS

Bacterial strains and Media

L. pneumophila AA100 is a virulent clinical isolate and has been described previously (Abu Kwaik, Eisenstein et al. 1993). The isogenic *dotA*, *htrA*, and *rib* mutants used in this study have also been previously described (Gao, Harb et al. 1997; Gao and Abu Kwaik 2000; Pedersen, Radulic et al. 2001). The three strains of *L. longbeachae* used (D 4968, D 4969, and D 4973) were clinical isolates from three outbreaks in the US (Oregon, Washington, California) and obtained from Dr. Barry Fields of the CDC, and have been passaged less than 3 times. The *L. longbeachae* strain ATCC 33462 was obtained from the American Type Culture Collection (ATCC). *L. pneumophila* and *L. longbeachae* were grown from frozen stocks on buffered charcoal yeast extract (BCYE) agar plates for 72 h before infection. The wild type *L. pneumophila*, *dotA* and *htrA* mutant strains used for the confocal studies harbored the plasmid pAM239, which encodes *gfp* (Pedersen, Radulic et al. 2001). The plates for the *gfp*-transformed AA100 were supplemented with 5µg/ml chloramphenicol and the plates for the *gfp*-transformed *dotA* strain were supplemented with 5µg/ml chloramphenicol and 50µg/ml kanamycin.

Tissue culture

U937 Macrophages-like cell lines and human monocyte-derived macrophages

The U937 macrophage-like cell line was maintained as described previously (Gao, Harb et al. 1997). At 48 h prior to infection, U937 cells were differentiated with phorbol myristate acetate (PMA) in either 96-well plate for studies of growth kinetics, or 24-well plate for confocal microscopy. MDM were isolated from single donor blood by the hypaque ficol method as previously described (Welsh, Summersgill et al. 2004). For both U937 and MDM, 10^5 and 5×10^5 cells were seeded in 96-well and 24-well plates, respectively. For infection of the cell monolayers, bacteria were re-suspended in RPMI 1640. The infection was carried out as described below for each experiment.

Murine Macrophage Cell lines

Immortalized macrophage cell lines were established by infecting bone marrow derived macrophages from A/J, C57BL/6 and BALB/c mice with the murine recombinant J2 retrovirus, as previously described (Blasi, Mathieson et al. 1985; Clemons-Miller, Cox et al. 2000).

Intracellular growth kinetics in U937 Macrophages

Monolayers of U937 macrophages were infected with the appropriate strains of *L. longbeachae* at MOI of 1 in triplicate. To synchronize the infection, the plates were centrifuged for 5 min at 1000 rpm using a centra GP8R Thermo IEC centrifuge. After 1 h of incubation in CO₂ at 37°C, the infected macrophages were washed 3 times with the culture medium to remove extracellular bacteria and incubated with 50µg/ml gentamicin for 1 h to kill the remaining extracellular bacteria. This was considered the zero (To) time point. The infected U937 macrophages were subsequently incubated for 12, 24, 48, 72

and 96 h. At the end of each time interval, the culture supernatant was removed and the cells lysed by addition of 200µl of sterile water for 10 min. The supernatant and the lysate were then combined, serial dilutions prepared and aliquots plated on BCYE to enumerate bacteria.

Intracellular growth kinetics in Murine Macrophage cell lines

Monolayer of immortalized murine macrophages was infected with the three different strains of *L. longbeachae* at MOI of 10 in triplicate as described above. The infected mouse macrophages were subsequently incubated for 8, 24, and 48, h. At the end of each time interval, the culture supernatant was removed and the cells were lysed by the addition of 200µl of sterile water for 10 min. The supernatant and the lysate were combined, serial dilutions were prepared and aliquots plated on BCYE to enumerate bacteria.

Antibodies and Laser scanning confocal microscopy

Confocal laser scanning microscopy and sample analysis was performed using mouse monoclonal anti-EEA1 (BD transduction), anti-LAMP-2 (H4B4) (Hybridoma gene bank), anti-Cathepsin D (BD transduction), anti-KDEL (Stressgen) anti-M6PR (Abcam, MA) and chicken anti-ATPase (Chemicon International). Anti-*L. longbeachae* rabbit polyclonal antibody was kindly provided by Dr. Barry Fields from the CDC. LAMP-2 antibody was use at 1:2000 dilutions, M6PR antibody at 1:100, ATPase antibody was 1: 1000, while KDEL and Cathepsin D antibodies were used at 1:200 dilutions. *L. longbeachae* polyclonal antibody was used at 1:4000 dilutions. Alexa flour

594-conjugated anti-mouse secondary antibody was used to stain the LAMP-2, KDEL and Cathepsin D. Alexa flour 488-conjugated secondary antibody was used to stain *L. longbeachae*. Texas Red Ovalbumin (TROV) was purchase from Molecular Probes. For determination of co-localization of *L. longbeachae* with EEA1, LAMP-2, M6PR, cathepsin D, TROV and KDEL, U937 cells were differentiated on 12-mm-diameter (0.13-to 0.17-mm-thick) circular glass cover slips (Fisher Scientific, Pittsburgh, Pa.) in 24-well culture plates. Multiplicity of infection of 10 was used for all the experiments for the laser scanning confocal microscopy (LSCM) except the 12 h infection where we determined the co-localization of replicating *L. longbeachae* with LAMP-2, where MOI of 1 was used to ensure approximate one bacterium per macrophage. The procedure for infection is as described for the growth kinetics except that no gentamicin was used after the infection period and washing off of the extracellular bacteria. Following infections, cells were washed three times with PBS and fixed in 4% paraformaldehyde for 30 min. Paraformaldehyde was removed by washing the wells three times with PBS. The U937 cells were permeabilized with 1% triton X 100 for the LAMP-2 experiment. For the KDEL and cathepsin D experiments, the cells were permeabilized with acetone for 5 min at -20 °C. All subsequent steps were performed at room temperature, and each step was followed by three washes in PBS. Blocking was performed with 3% bovine serum albumin in PBS for 1 h. For co-localization with Texas red ovalbumin(TROV) (molecular probes, Oregon), U937 cells were incubated with 100µg/ml TROV for 1.5 h followed by 30 min chase before the infection. The rest of the protocol was as described above. For the co-infection experiments, U937 macrophages were co-infected simultaneously with *L. longbeachae* strain D 4968 and *L. pneumophila* strain AA100 expressing GFP or its

isogenic *dotA* mutant also expressing GFP at MOI of 10 for *L. longbeachae* and *L. pneumophila* and 50 for the *dotA* mutant. As controls U937 macrophages were co-infected with *L. pneumophila* and its GFP expressing isogenic *dotA* and *htrA* mutants at the same MOI 10 and 50 respectively. To synchronize the infection, the plates were centrifuged for 5 min at 1000 rpm using a centra GP8R Thermo IEC centrifuge. After 1 h of incubation in CO₂ at 37°C, the infected macrophages were washed 3 times with the culture medium to remove extracellular bacteria. Infected cells were further incubated for 10 h and macrophages prepared for confocal as described above. ~ 100 infected cells were analysed from three cover slips for each experiment.

Transmission Electron Microscopy

To analyze the infection of U937 cells by *L. longbeachae* using transmission electron microscopy (TEM), monolayers were infected with *L. longbeachae* strain D 4968 on cover slips in 6-well plates at an MOI of 10 for 1 h followed by a 1 h of gentamicin treatment. At 6 h, 12 h and 24 h post infection, the infected U937 macrophages were processed as described previously (Gao, Harb et al. 1998). Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000/STEM electron microscope at 80 kV, as described previously (Gao, Harb et al. 1998).

Contact-dependent pore formation

Contact-dependent pore formation in membranes was determined by examining hemolysis of sheep red blood cells (sRBCs) by *L. longbeachae* strain D 4968 at an MOI

of 25 following 2 h of bacterial-sRBC contact, as previously described (Kirby, Vogel et al. 1998; Alli, Gao et al. 2000). *L. pneumophila* AA100 and its isogenic *dotA* and *rib* mutants GN229 (Alli, Gao et al. 2000) were used as positive and negative controls, respectively.

Infection of A/J, C57Bl/6 and Balb/c mice with *L. longbeachae*

Male and female pathogen-free, 6-8-week-old A/J, C57Bl/6 and Balb/c mice were used as approved by the University of Louisville IACUC. For the preparation of the intratracheal inoculation, *L. longbeachae* strains were quantified on BCYE agar plates that had been incubated for 48-72 h. The mice were injected intratracheally with 50µl containing 10⁶ bacteria as previously described (Gao, Stone et al. 1998). At 2 h, 24 h and 48 h post-inoculation, mice were humanely euthanized and the lungs were removed and bacteria enumerated on BCYE agar for 72-96 h as previously described (Gao, Stone et al. 1998). For the survival assays mice were observed for 14 days post-inoculation.

Histopathological analysis

Apoptosis in the lungs of A/J mice in response to *L. longbeachae* was assessed by confocal microscopy. At 24, 48 and 72h after inoculation with *L. longbeachae* strain D 4968, mice were humanely sacrificed. Before lung removal, the pulmonary vasculature was perfusing with 10 ml of saline containing 5mM EDTA, via the right ventricle. The excised lungs were then inflated and fixed in 10% neutral formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 µm) were cut and labeling of apoptotic cells was carried out using terminal

deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) with an in situ cell death detection kit, as recommended by the manufacturer (Roche, Indianapolis, IN). An analysis of apoptotic cells (TUNEL positive) was carried out using laser scanning confocal microscopy. On average, ten 0.2- μ m thick serial digital image sections were captured and stored for further analyses, with Adobe Photoshop (Adobe Photoshop, Inc.).

Determination of *naip5* expression by Real time PCR

Monolayers of immortalized macrophages were infected with the *L. longbeachae* strain D 4968 and *L. pneumophila* strain AA100 at MOI of 10 in 6 well plates as described above for the growth kinetics. The infected mouse macrophages were incubated for 2, 6, and 12, h. At the end of each time interval total RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) and digested with DNaseI (Ambion, Austin, TX) to remove chromosomal DNA. The remaining DNase I was inactivated by heating at 70°C for 5 mins. Five microgram of total RNA was then reverse transcribed into cDNA with SuperScript III RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA). Real time quantitative PCR was performed in an Opticon Continuous Fluorescence Detector (MJ Research, San Francisco, CA). The primer sequences for *naip5* were 5'-CTCCAGCCACTCTTCCTCAA-3' (forward primer) and 5'-ACCAGCCACAACCCTCTAAC-3' (Reverse primer), and the primer sequences for the β -Actin control were 5'- GATCTGGCACCACACCTTCT-3' and (forward primer) and 5'- GGGGTGTTGAAGGTCTCAAA-3'. The cDNA sample was amplified with DyNAmo SYBR Green qPCR Kits containing a modified *Thermus brockianus* DNA polymerase according to the manufacturer's instructions (New England Biolabs, Beverly,

MA). PCR conditions were 5 min at 94°C, 15 sec at 96°C and 15 sec at 72° for 30 cycles. The relative steady state level of *naip5* RNA was determined using the comparative C_T method according to manufacturer's software.

Caspase-3 activation

For detection of caspase-3 activity, U937 cells in 96-well plates were infected with either *L. longbeachae* strain D 4968, *L. pneumophila* strain AA100 or its isogenic *dotA* mutant at MOI of 10. At 6 h post-infection, caspase-3 activity in infected U937 cells was measured with a Fluorometric Caspase-3 Assay Kit (BioVision, Inc., CA) as described previously (Molmeret, Zink et al. 2004). Caspase-3 enzymatic activity was expressed in arbitrary fluorescent units (AFU) by using a fluorescent plate reader (Perkin-Elmer) with an excitation wavelength at 400nm and emission at 505nm.

For detection of active caspase-3 by confocal microscopy, U937 cells attached to glass coverslips in 24-well plates were infected with *L. longbeachae* strain ATCC 33462 (gfp), *L. pneumophila* Strain AA100 (gfp) or its isogenic *dotA* mutant at MOI of 10. For labeling of bacteria, cells were fixed with 4% paraformaldehyde (Sigma) for 30 min, permeabilized with 0.1% Triton X-100 (Sigma) on ice for 15 min, blocked with 3% bovine serum albumin (Sigma) for 1 h, incubated with rabbit polyclonal antiserum (raised against active caspase-3) for 1 h, and then incubated for 1 h with a goat anti-rabbit immunoglobulin G secondary antibody conjugated to Alexa red (Molecular Probes, Inc., Eugene, Oreg.).

TUNEL Assay

U937 cells attached to glass coverslips in 24-well plates were infected with *L. longbeachae* D 4968 and *L. pneumophila* A100 and its isogenic *dotA* mutant at MOI of 10. For labeling of bacteria, cells were fixed with 4% paraformaldehyde (Sigma) for 30 min, permeabilized with 0.1% Triton X-100 (Sigma) on ice for 15 min, blocked with 3% bovine serum albumin (Sigma) for 1 h, incubated with rabbit polyclonal antiserum (raised against *L. pneumophila* AA100 or *L. longbeachae*) for 1 h, and then incubated for 1 h with a goat anti-rabbit immunoglobulin G secondary antibody conjugated to Alexa red (Molecular Probes, Inc., Eugene, Oreg.). For labeling of apoptotic nuclei, the cells were then subjected to fluorescein isothiocyanate (FITC)-conjugated terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using an apoptosis detection kit in according to manufacturer's instructions (Boehringer Mannheim Corporation, Indianapolis, Ind.). Cells were examined with a Zeiss Axiophot Photomicroscope Leica TCS NT confocal laser scanning microscope. A minimum of 100 cells per sample was counted, and apoptosis was quantified as the percentage of apoptotic cells (TUNEL-positive nuclei) among all of the cells counted. Multiple independent samples were examined.

RESULTS

Growth kinetics of *L. longbeachae* in U937 and Murine macrophage cell lines.

We determined the ability of three *L. longbeachae* clinical isolates (D 4968, D 4969 and D 4973) of to replicate within human-derived U937 macrophages. Host cells were infected with *L. longbeachae* at MOI of 10 for 1 h, followed by gentamicin treatment for 1 h to kill extracellular bacteria, and the number of CFU enumerated at several time points. There was a robust intracellular growth of all three strains. By 96 h post infection there was 10^4 - 10^5 fold increase in the CFU, for all three strains. There were no major differences between the ability of these strains to replicate intracellularly (Fig 1A). However, pre-activation of macrophages with interferon- γ (IFN- γ) inhibited the growth of *L. longbeachae* strain D 4968 within U937 cells (Fig 1B). The ATCC strain 33462 of *L. longbeachae* replicated within macrophages similarly to the clinical isolates (Fig 1C). Therefore, *L. longbeachae* is capable of robust intracellular replication within quiescent but not in IFN- γ -activated macrophages.

Numerous reports have documented that *L. pneumophila* replicates in A/J mice but not in other inbred strains of mice (Hedlund, McGann et al. 1979; Yoshida and Mizuguchi 1986; Yamamoto, Klein et al. 1987; Yamamoto, Klein et al. 1988; Yamamoto, Klein et al. 1988; Yoshida, Goto et al. 1991; Miyamoto, Maruta et al. 1996; Izu, Yoshida et al. 1999). This is in contrast to many other *Legionella* species which can replicate in a variety of inbred mouse strains

(Miyamoto, Maruta et al. 1996; Izu, Yoshida et al. 1999). The ability of *L. pneumophila* to replicate in A/J mice is associated with polymorphism in the autosomal recessive gene *naip5* (Yoshida and Mizuguchi 1986; Yamamoto, Klein et al. 1988; Diez, Yaraghi et al. 2000; Growney and Dietrich 2000; Diez, Lee et al. 2003; Wright, Goodart et al. 2003). We examined whether polymorphism in the *naip5* gene was involved in the genetic susceptibility to infection by *L. longbeachae*.

To study the effect of *naip5* on the growth of *L. longbeachae* *in vitro*, immortalized bone marrow-derived macrophages from A/J, C57Bl/6 and Balb/c mice were infected with *L. longbeachae* clinical isolate D 4968 at MOI of 10 and the number of cfu was determined over a 48 h period. Interestingly, *L. longbeachae* replicated efficiently in macrophages from all three strains of mice examined (Fig 1). By 48 h post infection there was $\sim 10^4$ -fold increase in the number of cfu. *L. pneumophila* replicated only A/J mice derived macrophages, similar to *L. longbeachae*, consistent with previous observations (see below).

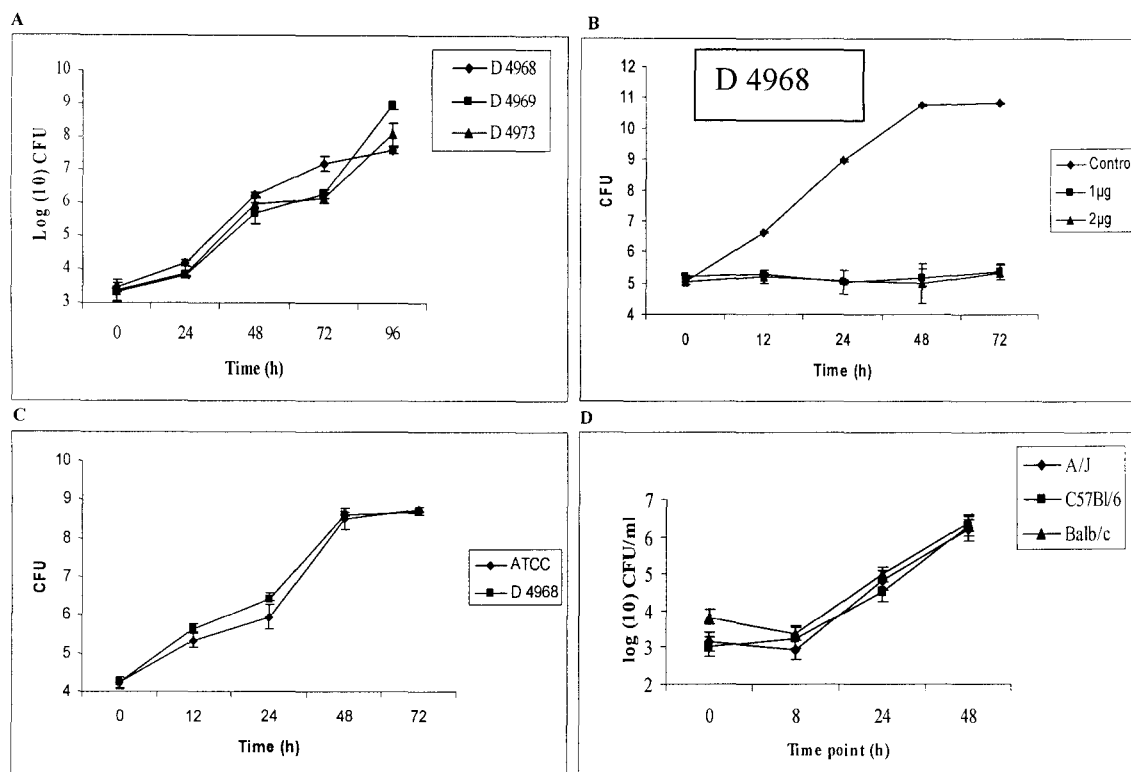


Figure 1. Intracellular proliferation of *L. longbeachae* within quiescent U937 and murine macrophage cell lines but not in IFN- γ activated U937 macrophages. (A) U937 Cells were infected with the three strains of *L. longbeachae* for 1 h at MOI of 10 followed by gentamicin treatment for 1 h, and the numbers of bacteria in the infected monolayers were determined at the indicated time points. (B) U937 cells were pretreated for 1 h with 1 or 2 μ g of IFN- γ and infected with strain D 4968 and the cfu was determined at the indicated time points post-infection. (C) U937 cells were infected with the clinical isolate D4968 and the ATCC strain 33476 at MOI of 10 and the cfu was determined at the indicated time points. (D) Murine macrophage cell lines were infected with *L. longbeachae* strain D4968 at MOI of 10 for 1 h, followed by gentamicin treatment for 1 h, and cfu was determined at the indicated time points. Results are representative of three independent experiments, and error bars represent standard deviation.

Growth phase-independent virulence of *L. longbeachae*

It is well documented that the ability of *L. pneumophila* to replicate in cells is totally dependent on the bacterial growth phase of the used as inoculum (Byrne and

Swanson 1998; Hammer and Swanson 1999; Bachman and Swanson 2001; Hammer, Tateda et al. 2002). The bacterium has coupled its conversion to stationary phase physiology with expression of virulence phenotype that promotes transmission to a new host. While post-exponential *L. pneumophila* evades endocytic fusion and replicates intracellularly, exponentially-grown *L. pneumophila* is completely defective in intracellular replication (Byrne and Swanson 1998), and this is mediated by the stringent response to starvation for amino acids (Byrne and Swanson 1998; Hammer and Swanson 1999; Bachman and Swanson 2001; Hammer, Tateda et al. 2002). Therefore, we examined whether the growth phase of *L. longbeachae* inocula had a similar effect on intracellular replication. *L. pneumophila* Philadelphia and AA100 strains showed growth phase-dependent intracellular replication, and with both strains, post exponential phase bacteria showed significantly more growth in MDMs compared to exponential phase bacteria ($p < 0.0001$) (Fig 2). In contrast, *L. longbeachae* replicated intracellularly to the same extent ($p = 0.06$) regardless of the growth phase of the bacteria used for infection in human monocyte-derived macrophages (Fig 2 C). It has previously been shown that post exponential *L. pneumophila* is motile while exponential phase *L. pneumophila* is not. This difference is also exhibited within macrophages (Byrne and Swanson 1998). Therefore, we examined the growth phase-dependent motility of *L. longbeachae*, and contrasted that to two *L. pneumophila* clinical isolates (Philadelphia and AA100). Our data showed that for the two *L. pneumophila* strains, less than 10% of exponential phase bacteria ($OD_{550} = 0.6$) were motile. In contrast, ~ 60-80% of the organisms from *in vitro*-grown post exponential phase ($OD_{550} = 2.0$) *L. pneumophila* strains were motile (data not shown), which is consistent with previous observations (Byrne and Swanson 1998).

Unlike *L. pneumophila*, almost 100% of *L. longbeachae* were motile at both exponential and post exponential phases *in vitro*, and when examined by phase contrast microscopy (data not shown). Taken together, our data showed that in contrast to *L. pneumophila*, motility and the ability of *L. longbeachae* to replicate intracellularly is totally independent of the bacterial growth phase.

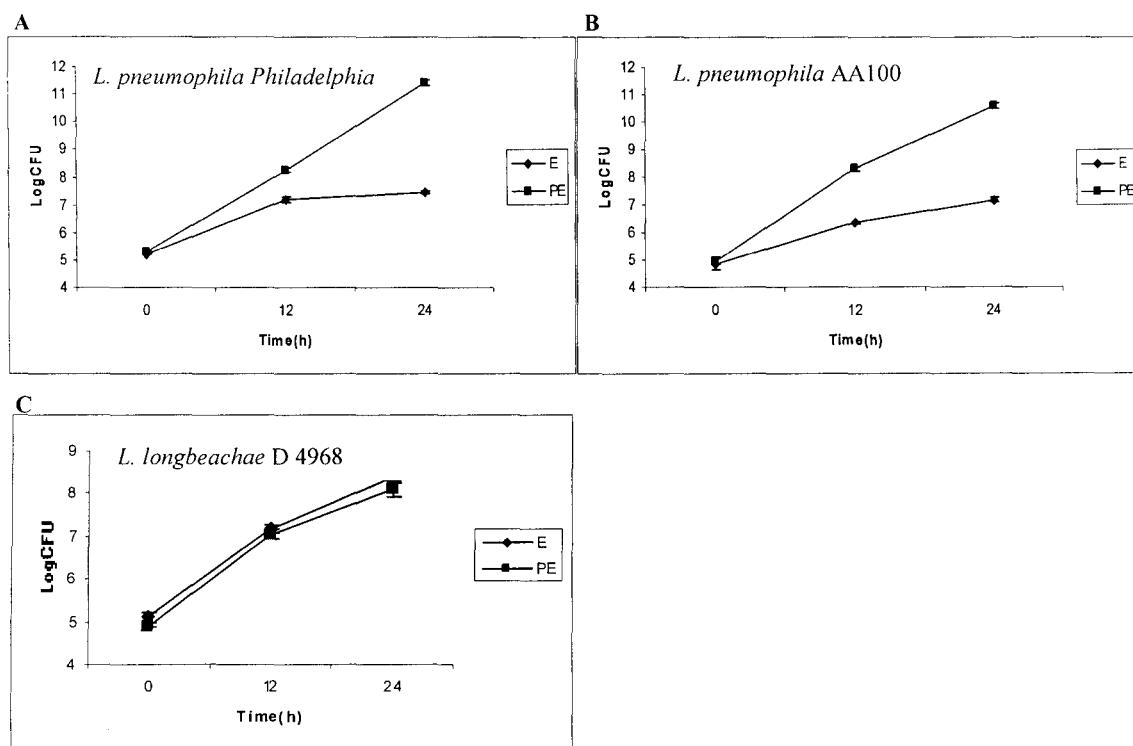


Figure 2. Growth phase-independent intracellular replication of *L. longbeachae*. Bacteria were grown in BYE to the exponential (E) or post-exponential (PE) phase and used to infect monocyte-derived macrophages (MDMs) as described in materials and methods. At the indicated time points, the MDMs were lysed and the cfu was enumerated. (A) *L. pneumophila* Philadelphia strain (B) *L. pneumophila* AA100 and (C) *L. longbeachae* D 4968. At 48h, there was no difference in the cfu of both E and PE *L. Longbeachae* in contrast to *L. pneumophila*. All results are representative of three independent experiments, and error bars represent standard deviation.

***L. longbeachae* is equally lethal to several inbred mice strains**

We determined whether *L. longbeachae* caused lung infection in the three inbred mice strains. First, we performed a lethality study in A/J mice to determine the survival of mice after inoculation with *L. longbeachae* and *L. pneumophila*. Different doses of *L. longbeachae* clinical isolate D 4968 and *L. pneumophila* strain AA100 were inoculated intratracheally into A/J mice to determine the number of bacteria needed to establish a lethal infection in mice. As low as 1×10^5 cfu of *L. longbeachae* were lethal to mice, and most of the mice (7/8) infected by 10^5 bacteria died by 5 days post inoculation (Fig 3).

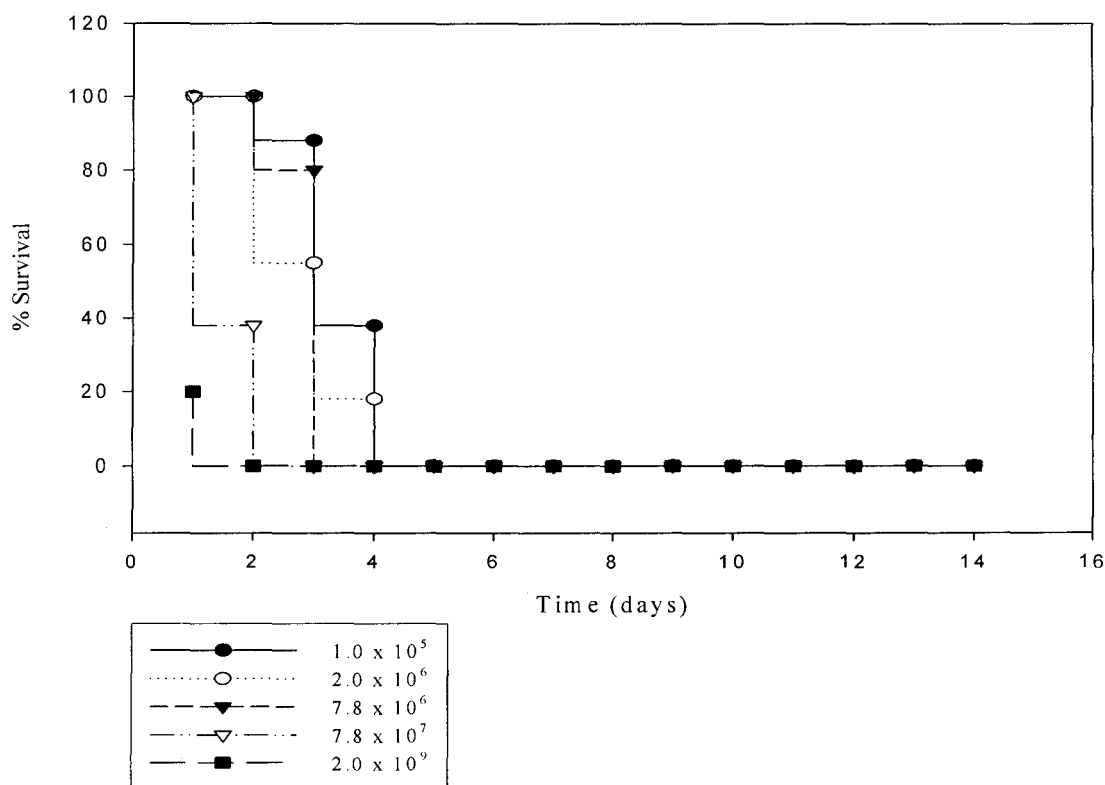


Figure 3. Lethality of A/J mice infected with different doses of *L. longbeachae*. Mice were infected intratracheally with different doses of *L. longbeachae* strain D 4968 and lethality of the mice was monitored over 14 days.

Unlike *L. longbeachae*, as high as 10^7 cfu of *L. pneumophila* were not lethal to A/J mice (Fig 4). Infection of A/J mice by 10^8 cfu of *L. pneumophila* caused only 30% lethality, where 3/10 mice succumb to infection. We conclude that *L. longbeachae* causes a more severe infection in A/J mice compared to *L. pneumophila* strain AA100

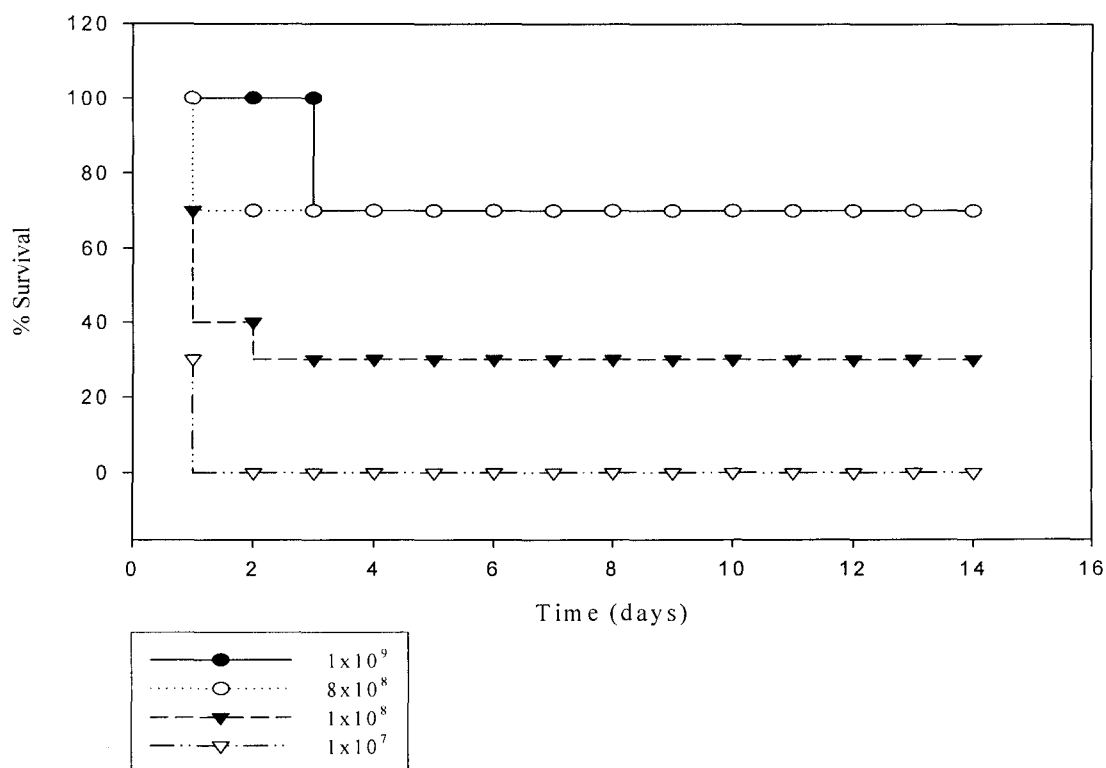


Figure 4. Lethality of mice infected with different doses of *L. pneumophila*. Mice were infected intratracheally with different doses of *L. pneumophila* strain AA100 and lethality of the mice was monitored over 14 days.

To examine lethality of *L. longbeachae* and *L. pneumophila* in the three strains of mice, A/J, C57Bl/6 and Balb/c were infected with 10^5 cfu of *L. longbeachae* clinical isolated D 4968. Lethality of *L. longbeachae* was similar for all 3 mouse strains (Fig 5).

In the C57Bl/6 and Balb/c mice, 4/5 and 6/7 mice, respectively, died by day 6 post-inoculation. Similarly, by day 5 post-inoculation, 7/8 A/J mice died. In contrast, *L. pneumophila* was not lethal to Balb/c or C57Bl/6 mice even when 10^9 cfu was inoculated, and all 10/10 animals survived (data not shown). As expected, *L. pneumophila* strain AA100 was lethal to A/J mice in a dose-dependent manner (table 2).

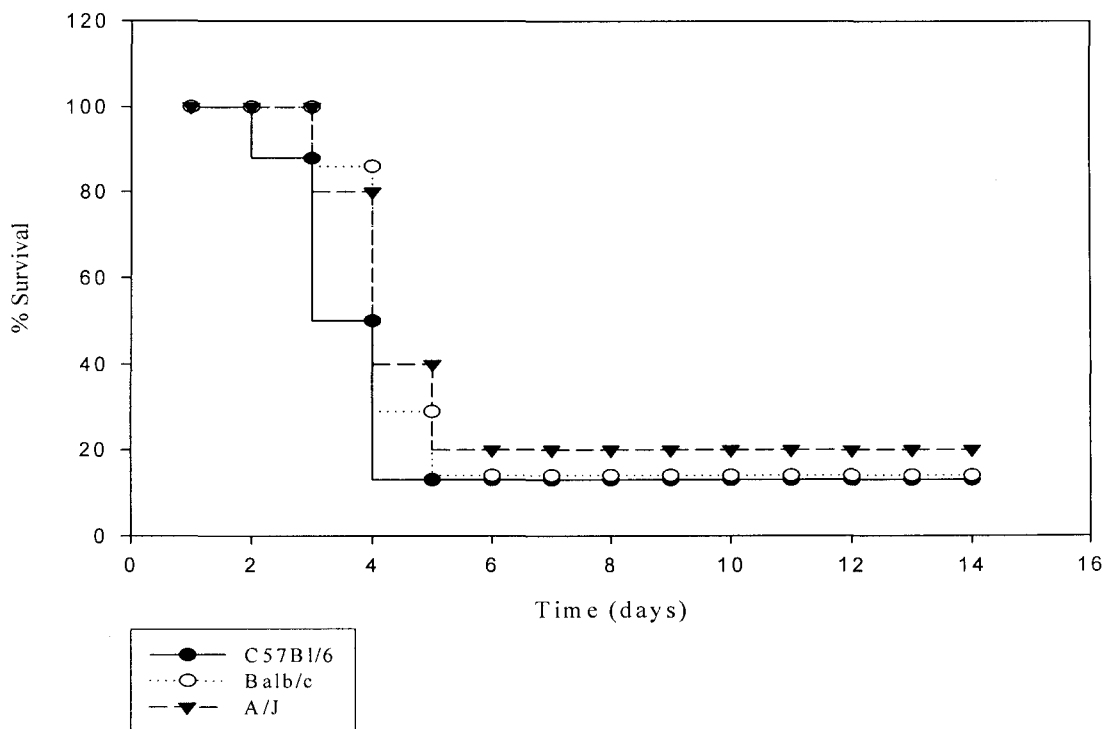


Figure 5. Lethality of different strains of mice infected intratracheally with *L. longbeachae*. A/J, C57Bl/6 and Balb/c mice were infected intratracheally with 1×10^5 of *L. longbeachae* strain D 4968 and lethality monitored over 14 days.

Intrapulmonary replication of *L. longbeachae* and *L. pneumophila* in inbred mice strains

To determine that lethality of mice was due to replication of *L. longbeachae* in the lungs, A/J mice were infected with 3 different doses of the *L. longbeachae* clinical isolate D 4968 and *L. pneumophila* strain AA100 and the number of cfu was determined over a 48 h period. As shown in Fig 2, all doses of *L. longbeachae* caused a productive pulmonary infection in A/J mice. Unlike *L. longbeachae*, the number of cfus was not increased in the lungs following infection by *L. pneumophila* when less than 10^6 bacteria were used for infection (data not shown). For all the infectious doses shown, there was about two log increases in the number of bacteria in the lungs from 2h to 48h and one log increase for *L. pneumophila* (Fig. 3).

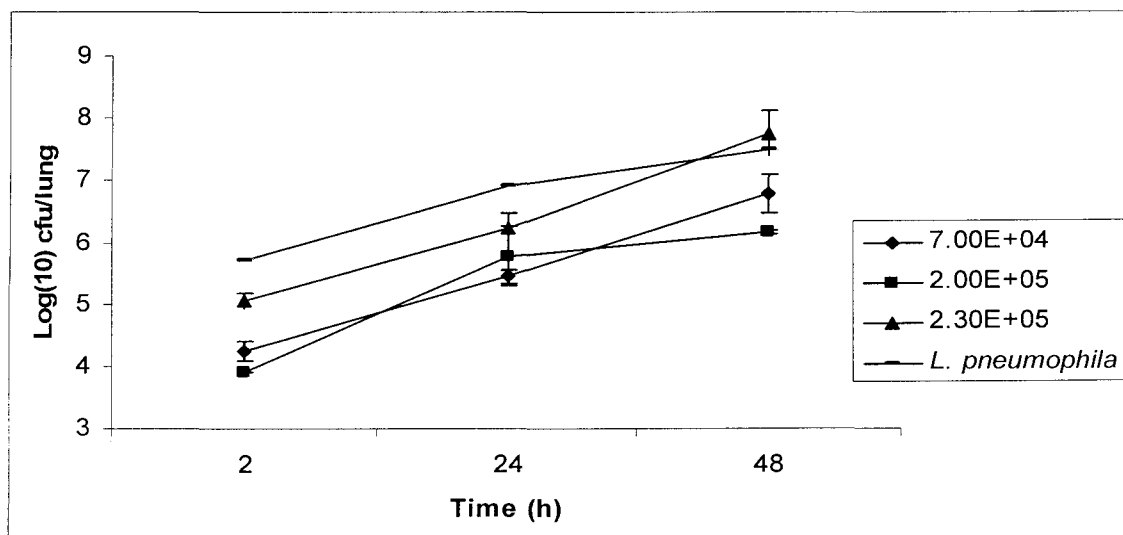


Figure 6. Infection of A/J mice by different doses of *L. longbeachae*. Replication of *L. longbeachae* strain D4968 in the lungs of A/J mice. A/J mice were infected with different doses and the number of cfu in the lungs was determined at the indicated time points. The control is *L. pneumophila* AA100 with inoculation dose of 1×10^6 cfus. Less than 1×10^6 cfus did not result in detectable intrapulmonary proliferation (data not shown). Results are representative of three independent experiments, and error bars represent standard deviation.

Next, A/J, C57Bl/6, and Balb/c mice were inoculated with $\sim 10^5$ cfus of *L. longbeachae* clinical isolates D 4968 and 10^6 cfus of *L. pneumophila* strain AA100 and the number of cfu in the lungs was determined over a 48 h period. (A 10^6 cfus of *L. pneumophila* strain AA100 was used since this is the minimal dose that results in intrapulmonary proliferation). The results indicated that *L. longbeachae* replicated equally well in the lungs of all three strains of mice ($p < 0.0001$). There was \sim a 1000-fold increase in the number of cfu over the 48 h period in all 3 strains (Fig 4 A). In contrast to *L. longbeachae*, *L. pneumophila* replicated only in A/J mice (Fig 4 B). Unlike growth in A/J mice where the cfu of *L. pneumophila* showed about 1000 fold increase over a 48 h period, the cfu in C57Bl/6 and Balb/c mice increased by only 2-4 fold between 2 and 24 h and dropped slightly by 48 h post infection (Fig 4 B). The p value for the difference between A/J and C57Bl/6 or Balb/c was greater than 0.05.

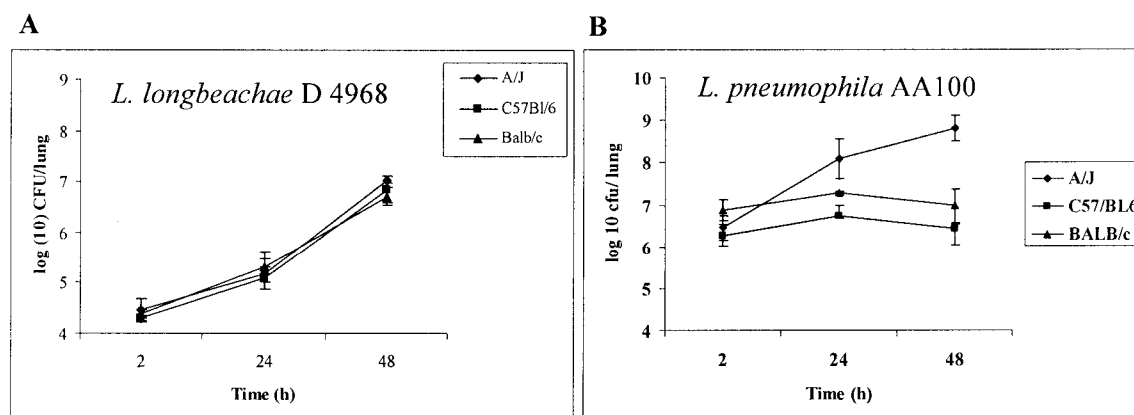


Figure 7. A/J, C57Bl/6 and Balb/c inbred mice strains are equally susceptible to infection of *L. longbeachae*. Intrapulmonary replication of *L. longbeachae* and *L. pneumophila* in A/J, C57Bl/6, and Balb/c mice was determined. The three strains of mice were inoculated with (A) 1×10^5 cfu of *L. longbeachae* strain D 4968; (B) 10^6 cfu of *L. pneumophila* strain AA100. Lungs were harvested and the number of cfu in the lungs was determined at the indicated time points. Results are representative of three independent experiments, and error bars represent standard deviations.

Susceptibility of inbred mice strains to *L. longbeachae* is not strain-dependent

To confirm that genetic susceptibility of the three mice strains was not limited to the D 4968 clinical isolate, infection by another clinical isolate, D 4973, was examined. A/J, C57Bl/6, and Balb/c mice were infected with 1.2×10^5 cfu of clinical isolate D 4973 and lethality was monitored over 14 days. Lethality was found to be similar to that of the D 4968 isolate (data not shown). In addition, replication of *L. longbeachae* clinical isolate D 4973 in the lungs of A/J, C57Bl/6 and Balb/c mice was determined. The mice strains were inoculated intratracheally with 1.2×10^5 bacteria and bacterial replication was determined over a 48 h period. The clinical isolate D 4973 replicated in all 3 strains of mice but with higher level of growth in A/J mice compared to strain D 4868 at 48 h post infection ($p < 0.05$) (Fig 5), indicating slight differences between these two clinical isolates. Taken together, we conclude that in contrast to *L. pneumophila*, polymorphism in the *naip5* allele has no effect on genetic susceptibility of mice to infection by *L. longbeachae* strains.

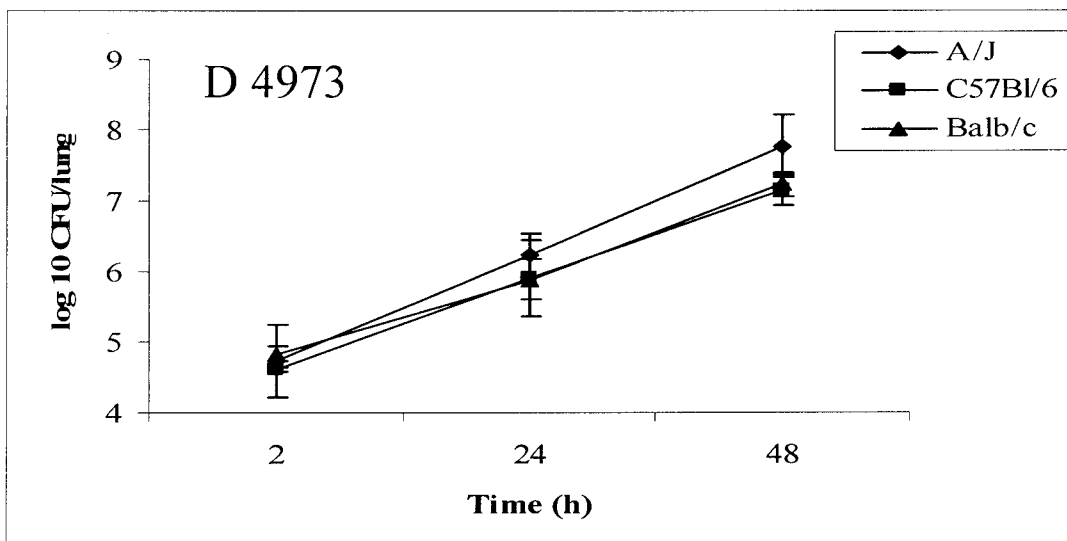


Figure 8. Inbred mice strains are equally susceptible to infection by different strains of *L. longbeachae*. Intrapulmonary replication of *L. longbeachae* strain D 4973 in A/J, C57Bl/6, and Balb/c mice was determined. The three strains of mice were inoculated with 1.2×10^5 cfu of *L. longbeachae* strain D 4973. Lungs were harvested and the number of cfu in the lungs was determined at the indicated time points. Results are representative of three independent experiments, and error bars represent standard deviation.

Acquisition of the early endosomal antigen 1 (EEA1) by the *L. longbeachae* phagosome

Majority of the work that has examined trafficking of *Legionella* has been limited to *L. pneumophila*. We initiated studies of intracellular trafficking of *L. longbeachae* within U937 macrophages. The cells were infected with the *L. longbeachae* strain D 4968 at MOI 10 and the cells were fixed and labeled at 5 and 15 min post-infection with antibody against EEA1, which is an early endosomal marker. Co-localization of *L. longbeachae* with EEA1 was determined by confocal laser scanning microscopy. GFP-transformed *L. pneumophila* and its isogenic *dotA* mutant were used as negative and positive controls, respectively. The data showed that *L. longbeachae* co-localized with the early endosomal marker EEA1. At 5 or 15 min post-infection, 20% of *L. longbeachae*-containing phagosomes co-localized with EEA1 similar to the 23% co-localization for the *dotA* mutant of *L. pneumophila*. In contrast, only 3% of WT *L. pneumophila* phagosomes co-localized with EEA1 (Fig 6 A and B). The small percentages of *L. longbeachae* and *L. pneumophila dotA* mutant phagosomes that co-localized with EEA1 are a reflection of the transient nature of association of this marker with dynamic endocytic vesicles.

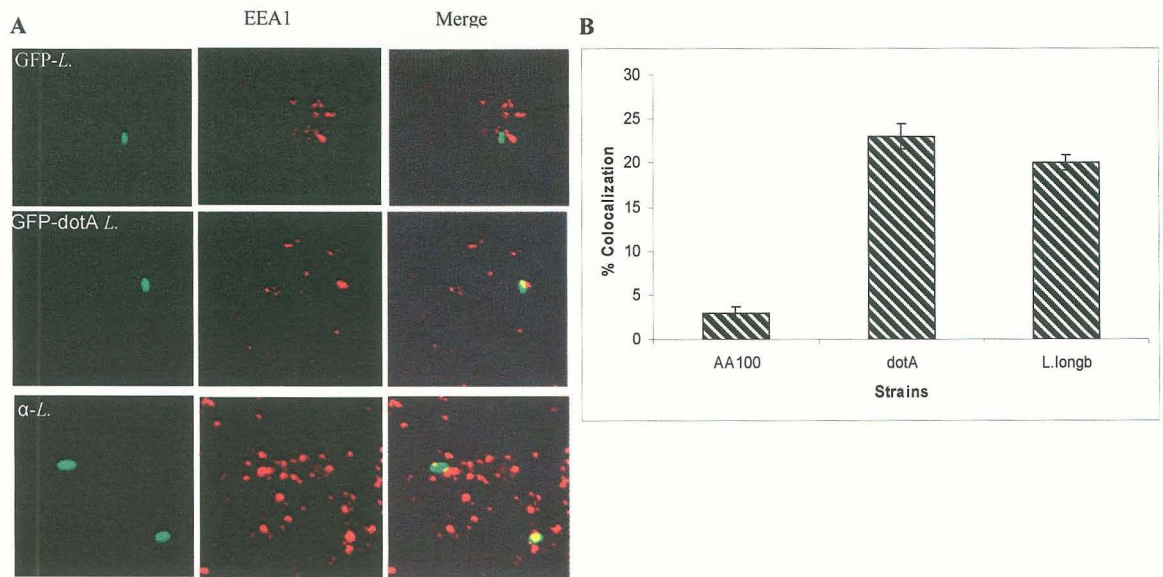


Figure 9. The *L. longbeachae* phagosomes co-localize with the early endosomal marker EEA1. (A) Representative confocal microscopy images of U937 macrophages infected with *L. longbeachae* strain D 4968 at MOI of 10 and co-localization with EEA1 was determined at 15 min post-infection. (B) Quantification of and co-localization with EEA1 was based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

Acquisition of the late endosomal markers LAMP-2 and M6PR by the *L. longbeachae* phagosome

When examined for co-localization with late endosomal markers, 82% of *L. longbeachae*-containing phagosomes co-localized with LAMP-2 similar to the *dotA* mutant of *L. pneumophila* (Fig. 7A and C). In contrast to *L. longbeachae*, ~30% of *L. pneumophila* AA100 co-localized with LAMP-2 (Fig 7 A and C), consistent with numerous previous observations. In addition to LAMP-2, we determined co-localization with the late endosomal marker Mannose-6-phosphate receptor (M6PR). Similar to LAMP-2, ~70% of *L. longbeachae* co-localized with the M6PR similar to the *dotA*

mutant that showed about 70% co-localization. In contrast, only 10% of *L. pneumophila* co-localized with the M6PR (Fig 7 B and C). To verify that maturation of the *L. longbeachae* containing phagosome to a late endosome-like compartment is not restricted to the clinical isolate, co-localization of the ATCC strain 33462 with LAMP-2 was determined. Similar to the clinical isolate, more than 85% of the ATCC strain phagosomes co-localized with the LAMP-2 marker (data not shown).

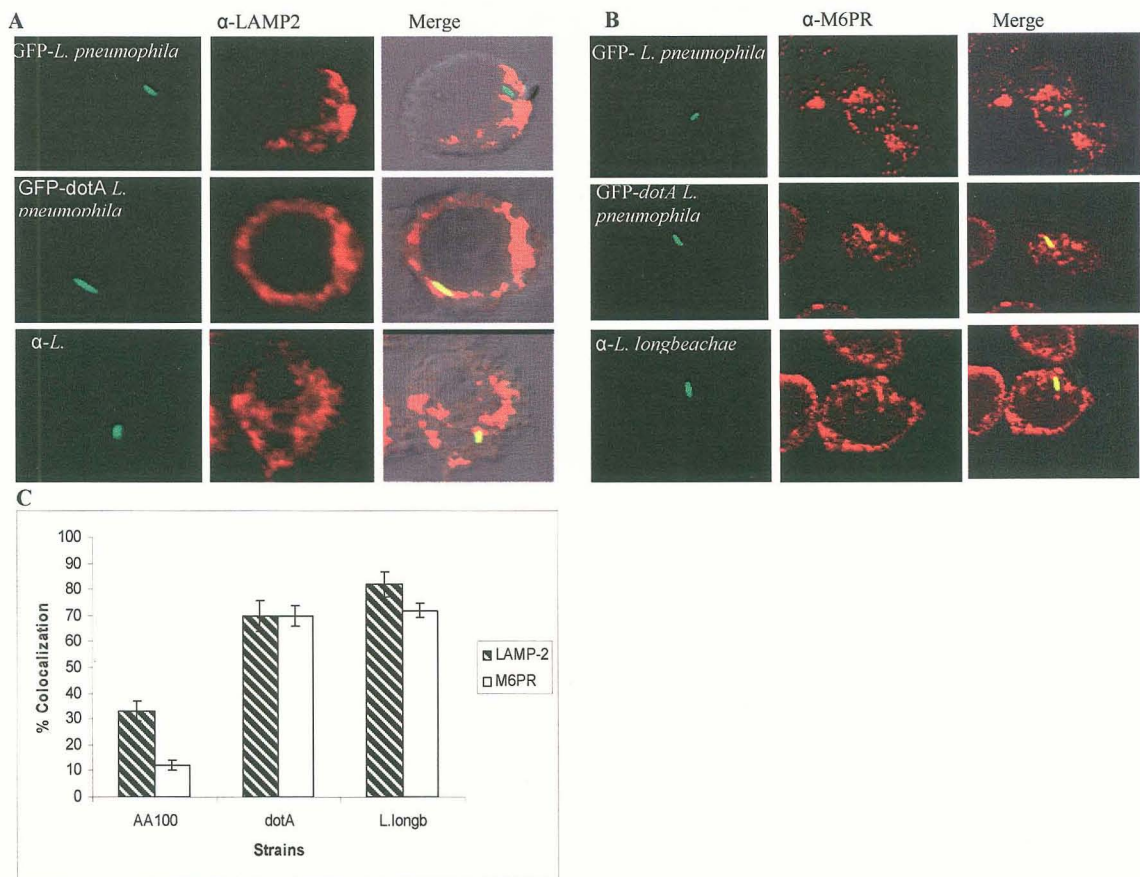


Figure 10. Co-localization of the *L. longbeachae*-containing phagosome with LAMP-2 and M6PR.

Representative confocal microscopy images of infected U937 macrophages, where co-localization of the phagosome with LAMP-2 (A) and M6PR (B) was determined at 1 h post-infection using MOI of 10. (C) Quantification of co-localization with LAMP-2 and M6PR was based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

Exclusion of the vacuolar ATPase proton pump by *L. longbeachae*

During endocytic maturation of phagosomes harboring particles that are destined to be degraded in the lysosomes, the phagosome becomes acidified through the action of the vacuolar ATPase (vATPase) proton pump that is acquired by the phagosome upon its maturation into a late endosome-like stage. Having established that *L. longbeachae* acquires late endosomal markers, we then determined if the *L. longbeachae* containing phagosome acquires the vATPase proton pump. The results showed that 62% of *L. longbeachae* containing phagosomes excluded the vATPase at 30 min post infection. This is similar to the *L. pneumophila* containing phagosome, where 63% of the phagosomes excluded the vATPase protein. In contrast, only ~35% of the phagosomes harboring formalin killed *L. longbeachae* excluded with the vATPase marker (Fig 8 A and B).

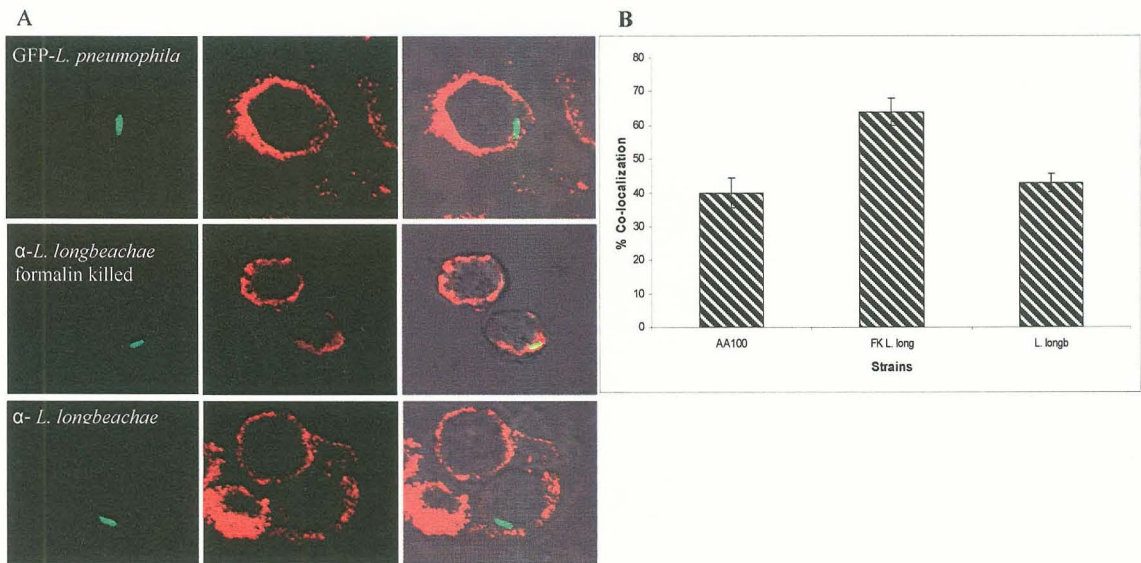


Figure 11. The *L. longbeachae* phagosome excludes the vATPase pump. Representative confocal microscopy image of U937 macrophages at 1 h post infection to examine co-localization of *L. longbeachae* and *L. pneumophila* within with the vATPase marker (A). (B) Quantification co-localization with the

vATPase marker was based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

Exclusion of the lysosomal markers Cathepsin D and Texas Red Ovalbumin by the *L. longbeachae* phagosome

Next, we determined if the *L. longbeachae* phagosome was co-localized with the lysosomal marker Cathepsin D at 1 h after infection. Our data showed that more than 70% of *L. longbeachae* phagosomes excluded cathepsin D (Fig 9 A and C). For the *dotA* mutant of *L. pneumophila* or formalin-killed *L. longbeachae*, used as positive controls, 15-25% of the phagosomes excluded Cathepsin D, while 85% of wild-type *L. pneumophila* excluded Cathepsin D (Fig 9 A and C, and data not shown). In addition to Cathepsin D, we determined co-localization of *L. longbeachae* with the lysosomal tracer TROV. The lysosomes of macrophages were preloaded with TROV and chased for 30 min prior to infection. At 1 h post-infection more than 75% of *L. longbeachae* did not co-localize with TROV (Fig 9 B and D). Similarly, more than 75% of wild-type *L. pneumophila* did not co-localize with TROV. In contrast, about 30% of formalin-killed *L. longbeachae*, which was used as a positive control, failed to localize to a TROV-containing phagosome (Fig. 9 D). To verify that the exclusion of lysosomal markers was not restricted to the clinical isolate, co-localization of the ATCC strain 33462 phagosomes with Cathepsin D was determined. Similar to the clinical isolate, ~70% of the ATCC 33462 strain phagosomes excluded Cathepsin D (data not shown). Taken together, the above results indicate that biogenesis of *L. longbeachae* phagosome is distinct from *L. pneumophila*. Unlike *L. pneumophila* whose phagosome is isolated from

the endocytic pathway, *L. longbeachae* resides in a phagosome with characteristics of a late endosome-like compartment that does not fuse to the lysosomes.

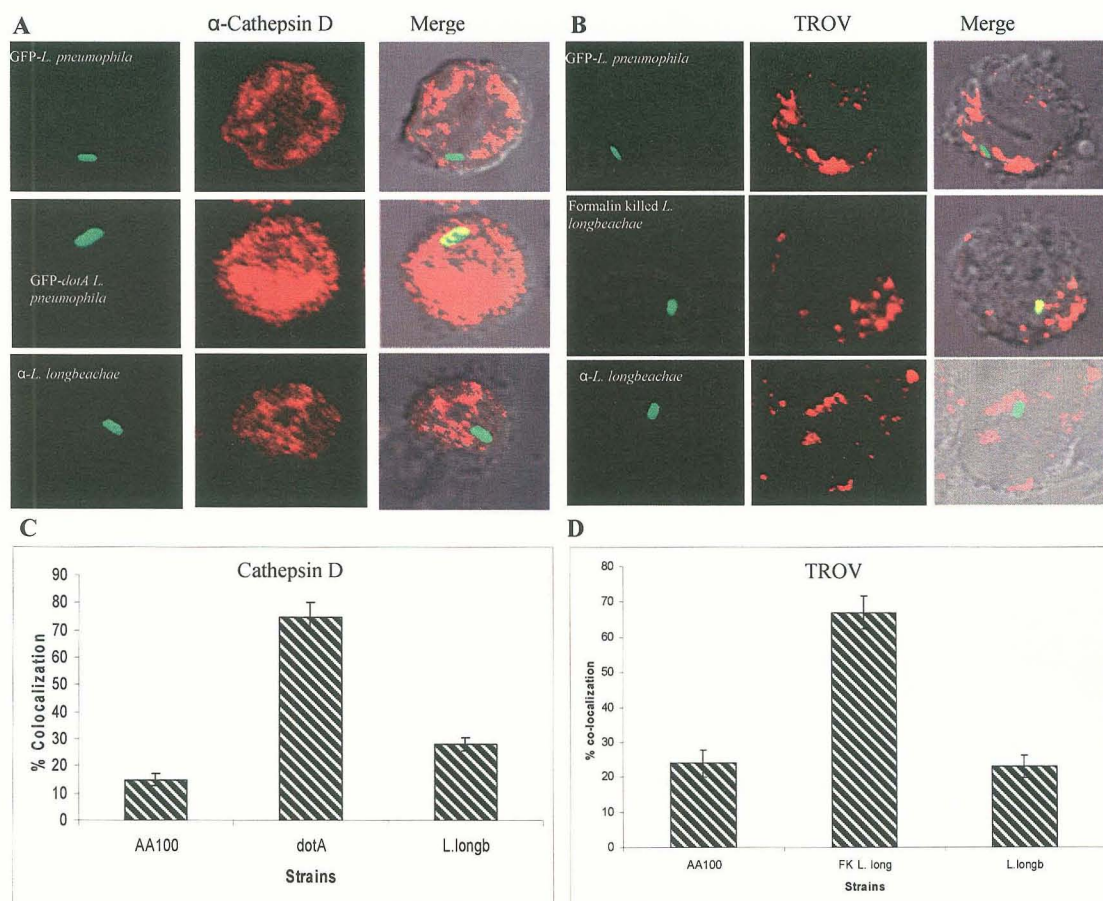


Figure 12. The *L. longbeachae* phagosome does not co-localize with Cathepsin D or TROV. Representative confocal images of infected U937 macrophages to determine co-localization with Cathepsin D (A) and (B) TROV. (C) and (D) show the quantification of co-localization with Cathepsin D and TROV based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

Replication of *L. longbeachae* in a late endosome-like compartment

Since the *L. longbeachae* containing phagosome co-localized with several late endosomal markers during the early stages of infection, we determined if bacteria at a

later time point also resided within late endosome-like phagosomes. Macrophages were infected with *L. longbeachae* strain D 4968 at MOI of 1 for 1h, gentamicin-treated for 1h, and the cells were labeled for LAMP-2 at 12 h post-infection (exponential replication). The data showed that 63% of replicative phagosomes were LAMP-2 positive. Unlike *L. longbeachae*, only 25% of replicative phagosomes of *L. pneumophila* co-localized with LAMP-2 (Fig 10 A and B). We conclude that the majority of the replicative *L. longbeachae* phagosomes are Lamp2-positive, indicating a late endosome-like characteristic.

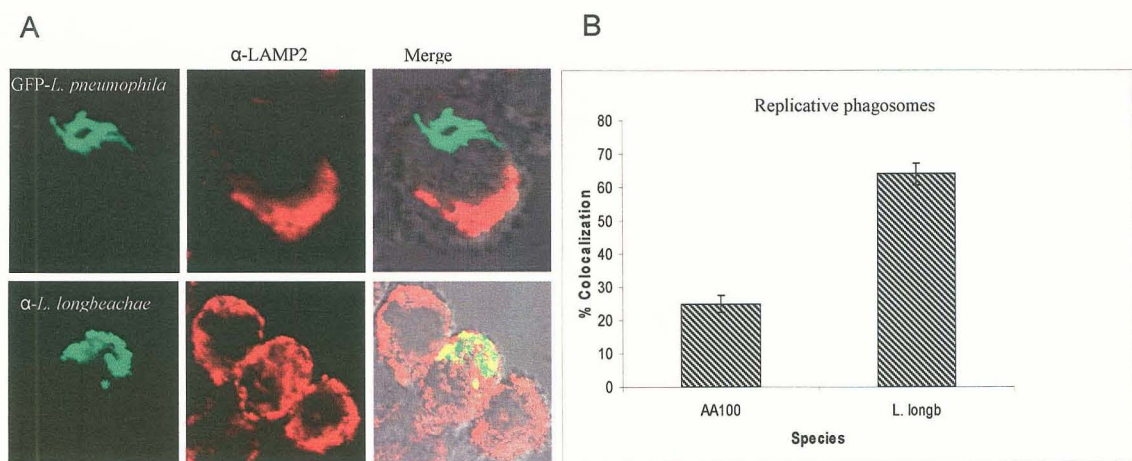


Figure 13. *L. longbeachae* replicates in a LAMP-2 positive phagosome. Representative confocal microscopy images of U937 macrophages infected with *L. longbeachae* or *L. pneumophila* for 1h, gentamicine-treated, and examined for Co-localization with LAMP-2 (A). (B) Quantification of Co-localization of the replicative phagosomes with LAMP-2 was based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

***L. longbeachae* is targeted to the RER**

L. pneumophila replicates in a phagosome surrounded by a membrane derived from the rough endoplasmic reticulum (RER). In contrast, *L. micdadei* phagosomes do not recruit the ER (Gao, Susa et al. 1999). The Dot/Icm type IV secretion system of *L. pneumophila* is central to remodeling of the phagosome into an ER-derived compartment. To determine whether *L. longbeachae* is targeted to the ER, both confocal microscopy and Transmission electron microscopy (TEM) were used. For the confocal microscopy studies, monolayers of U937 macrophages were infected for 1h, washed, and incubated at 37°C for an additional 1 h. *L. longbeachae* was labeled with rabbit polyclonal antiserum and anti-KDEL monoclonal antibody was used to label the ER. *L. pneumophila* and its *dotA* mutant were used as positive and negative controls, respectively. At 2 h post infection, 74% of *L. longbeachae* showed co-localization with the KDEL (Fig 11 A and B). *L. pneumophila* AA100 showed 64% co-localization with the KDEL while the *dotA* mutant showed only 23% co-localization (Fig 11 A and B). Similar to the clinical isolate, the ATCC strain 33462 of *L. longbeachae* acquired the ER with about 70% of the phagosome co-localized with the KDEL marker (data not shown).

To analyze recruitment of the ER to the *L. longbeachae* phagosome by TEM, infected macrophages were analyzed at 6 h and 12 h post infection. The results showed that at 6 h post-infection, the phagosome of *L. longbeachae* is surrounded by the RER, which persisted through 12 h post-infection (Fig 11 C). Taken together, the results show that the *L. longbeachae* phagosome has a late endosome-like characteristic and is targeted to the RER, which is a unique trafficking compared to *L. pneumophila*.

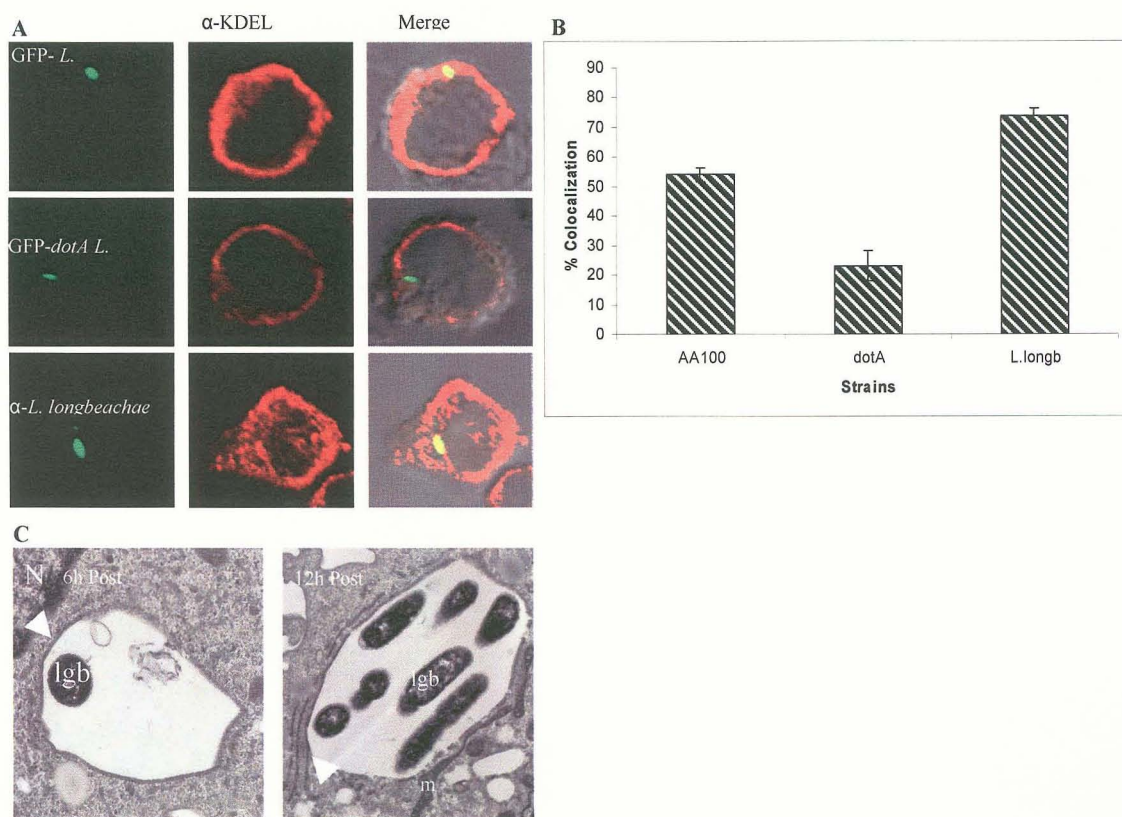


Figure 14. The *L. longbeachae* phagosome is surrounded by the RER. (A) Representative confocal microscopy images of U937 macrophage infected with *L. longbeachae* or *L. pneumophila* at MOI of 10 and co-localization with the ER marker KDEL was determined at 1 h post infection. (B) Transmission electron micrographs of *L. longbeachae* infected U937 macrophages at 6h and 12h post-infection. Lgb is for *L. longbeachae*, N is for Nucleus, and M for mitochondria. Arrowheads indicate the ER at the *L. longbeachae* phagosome. (C) Quantification of co-localization of the bacterial phagosome with the KDEL marker was based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

L. longbeachae* replicates in communal phagosomes with *L. pneumophila

Due to distinct biogenesis of the phagosomes harboring *L. longbeachae* and *L. pneumophila* during early stages, we hypothesized that the two organisms replicated in distinct intracellular niches and may not be able to replicate together in communal

phagosomes established and remodeled by either of the two species. To test this hypothesis, we co-infected macrophages with both species and determined whether both species can replicate in communal phagosomes. We used co-infection of wild type *L. pneumophila* and the two isogenic mutants, *dotA* or *htrA* as positive and negative controls, respectively. It has been shown that unlike the *htrA* mutant, which cannot be rescued by the wild type bacteria when they both reside in a communal phagosome (Pedersen, Radulic et al. 2001), the *dotA* mutant is rescued by the wild type *L. pneumophila* when they both reside in a communal phagosome (Coers, Monahan et al. 1999). In all our co-infections, only 4-8% of the phagosomes were communal phagosomes harboring the two different organisms used in the co-infection. These experiments showed that when both wild type *L. pneumophila* and *L. longbeachae* reside in a communal phagosome, both species are able to replicate (Fig. 12 A and B). There were equivalent numbers of bacteria for both species in the communal phagosomes harboring both replicating species (Fig 12). In the control co-infection of *L. pneumophila* and its *dotA* mutant, replication of the *dotA* mutant was rescued in communal phagosomes containing the wild type strain (Fig 12). Control co-infection with *L. pneumophila* and its *htrA* mutant showed failure of the wild type strain to rescue the *htrA* mutant in communal phagosomes (Fig 12), consistent with previous observations (Pedersen, Radulic et al. 2001). The data showed both *L. pneumophila* and *L. longbeachae* replicated in a communal phagosome.

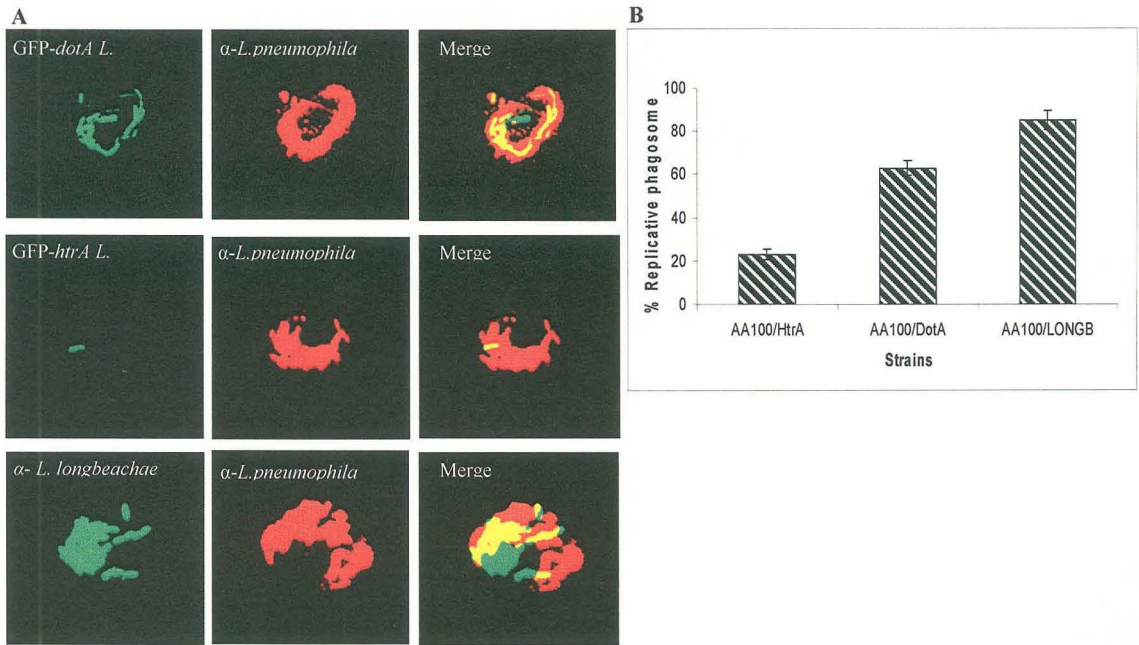


Figure 15. Replication of *L. pneumophila* and *L. longbeachae* within communal phagosomes. U937 macrophages were co-infected for 1 h followed by incubation for 12h. Co-infections with WT *L. pneumophila* and its isogenic *dotA* or *htrA* mutants were used as controls for rescue and no rescue, respectively, of the mutant to replicate within communal phagosomes harboring the WT strain of *L. pneumophila*. (A) Representative confocal microscopy images of the co-infections. (B) Quantification of the replicative communal phagosomes was based on analysis of 100 infected cells from three different coverslips. All results are representative of three independent experiments, and error bars represent standard deviation.

We then asked whether *L. longbeachae* can rescue the *dotA* mutant of *L. pneumophila* when both reside in communal phagosomes. To answer this question, we co-infected U937 macrophages with *L. longbeachae* and the *dotA* isogenic mutant of *L. pneumophila*. The data showed that in 80% of the communal phagosomes, *L. longbeachae* rescued the *dotA* mutant for intracellular replication (Fig 13). When the

dotA mutant resides alone in a phagosome, it does not replicate. We conclude that *L. pneumophila* can replicate in a phagosome established by *L. longbeachae*.

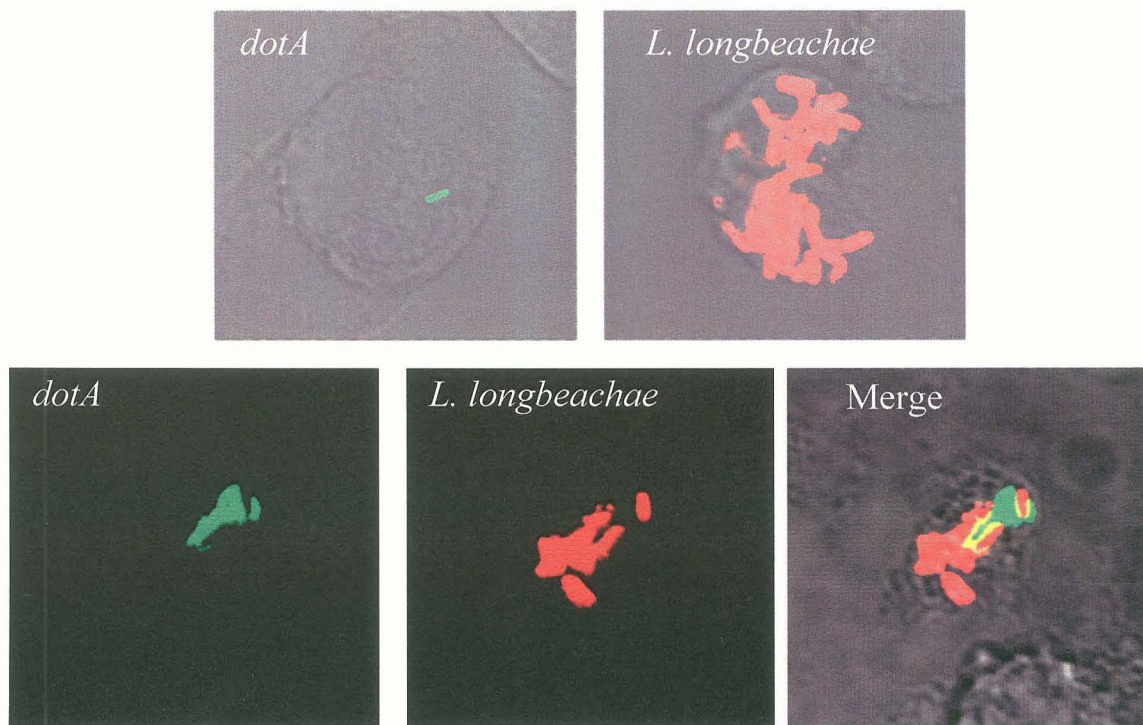


Figure 16. *L. longbeachae* rescues the *dotA* mutant of *L. pneumophila* for intracellular replication within communal phagosomes. U937 macrophages were co-infected with *L. longbeachae* and the *dotA* mutant of *L. pneumophila* for 1 h followed by incubation for 12h. Note that when the *dotA* mutant is alone in the phagosome it does not replicate. Approximately 80% of the communal phagosomes harboring *L. longbeachae* and the *dotA* mutant of *L. pneumophila* showed replication of the *dotA* mutant. The results are representative of three independent experiments.

***L. Longbeachae* egress into the cytoplasm during late stages of infection**

The pore-forming activity of *L. pneumophila*, which is triggered at the post-exponential phase, has been shown to contribute to cytotoxicity and the ability of the organism to egress from the host cell after cessation of intracellular replication (Byrne and Swanson 1998; Kirby, Vogel et al. 1998; Alli, Gao et al. 2000; Gao and Abu Kwaik

2000; Molmeret, All et al. 2002; Molmeret, Alli et al. 2002). Bacterial egress begins with a disruption of the phagosomal membrane at about 12 h post-infection leading eventually to residence of the bacteria in the cytoplasm before lysis of the plasma membrane and exit from the host cell (Molmeret, Bitar et al. 2004). Therefore, we examined integrity of the *L. longbeachae* containing phagosome during late stages of infection of macrophages at the ultrastructural level. Our results showed that whereas most bacteria are in intact phagosomes by 6 h post-infection, over 80% of the infected cells had disrupted phagosomes by 12 h post infection. By 24 h post infection, about >90% of the infected cells contained bacteria that were in the cytoplasm among cytoplasmic vesicles with no intact limiting phagosomal membrane (Fig 14 A and B), similar to *L. pneumophila* (Molmeret and Abu Kwaik 2002; Molmeret, All et al. 2002; Molmeret, Alli et al. 2002).

To examine whether *L. longbeachae* expressed a pore-forming activity, contact-dependent hemolysis of sRBC assays were performed for the three clinical isolates of *L. longbeachae* (Fig 14 C). The *L. pneumophila rib* mutant GN229, which is defective in the pore-forming activity (Molmeret, All et al. 2002; Molmeret, Alli et al. 2002) was used as a negative control in this assay while wild type *L. pneumophila* was the positive control. The data showed that unlike *L. pneumophila*, none of the three clinical isolates of *L. longbeachae* possessed any detectable pore-forming activity, similar to the GN229 mutant negative control.

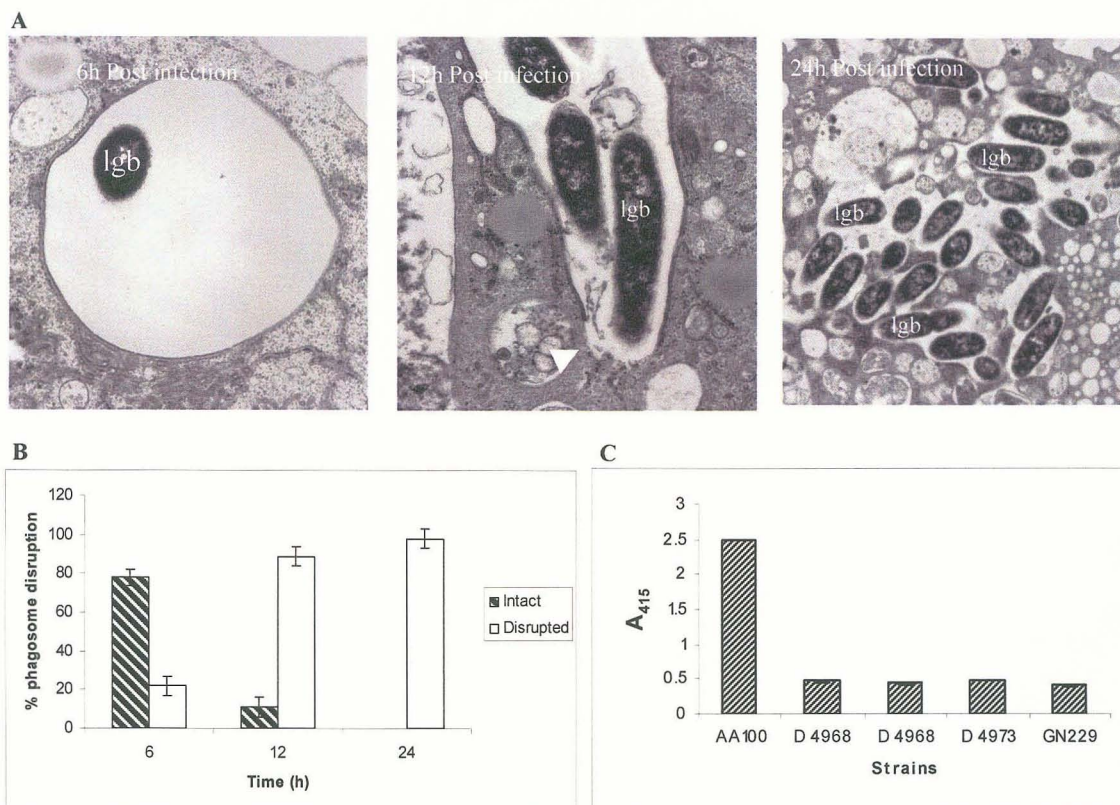


Figure 17. *L. longbeachae* egress from the phagosome during late stages of infection. U937 macrophages were infected with *L. longbeachae* for 1 h and at the indicated time points infected cells were analyzed TEM to determine integrity of the phagosomal membrane. (A) Representative images of TEM showing intact phagosomes at 6h and disruption of the phagosomal membrane and bacterial egress into the cytoplasm at 12-24h post-infection. (B) Quantification of integrity of the phagosome was based on analysis of 100 infected cells from three different blocs. (C) Contact-dependent hemolysis of sRBCs measured by absorbance of the released hemoglobin at a wavelength of 415. All results are representative of three independent experiments, and error bars represent standard deviation.

Expression of *naip5* in C57Bl/6 and A/J mice

Previous studies have shown that when expression of the *naip* family of genes (~8 genes) is examined as a group, their collective expression is induced after phagocytosis of *L. pneumophila*, *Salmonella typhimurium*, or latex beads (Molmeret, Zink et al. 2004),

but whether *naip5* is one of the induced genes is not known. In addition, A/J mice macrophages express less Naip proteins than C57Bl/6 mice, but whether Naip5 was partially or exclusively responsible for the different levels of *naips* is not known (Molmeret, Zink et al. 2004). Since polymorphism in the *naip5* allele did not have any effect on the ability of *L. longbeachae* to replicate in mice, we determined the level of *naip5* expression in A/J and C57Bl/6 macrophages during infection with *L. longbeachae* and contrasted it to *L. pneumophila*. Since *naip5* is the only *naip* gene among the ~8 *naip* genes that confers susceptibility to *L. pneumophila*, we focused our analysis exclusively on *naip5* expression. Bone marrow-derived macrophages from both mice strains were infected with *L. longbeachae* clinical isolate D 4968 or *L. pneumophila* strain AA100 for 2, 6, and 12 h. The mRNA was isolated from infected A/J and C57Bl/6 mice macrophages and the level of *naip5* expression was determined by Real Time PCR. mRNA of β -actin was used as the internal control. We determined the ratio of the expression of *naip5* in infected macrophages versus uninfected macrophages (Fig. 15A and B) as well as the ratio of C57Bl/6 infected macrophages versus A/J infected macrophages (Fig. 15C). The results showed that there was higher level of expression of *naip5* in C57Bl/6 mice macrophages compared to A/J mice macrophages. In uninfected bone marrow-derived macrophages, *naip5* was 2.6 fold higher in C57Bl/6 compared to A/J mice macrophages. At 2 h post infection, both *L. pneumophila* and *L. longbeachae* infected A/J and C57Bl/6 mice macrophages showed ~1.6 fold increase in *naip5* expression compared to uninfected macrophages. By 6 h post infection, the level of expression of *naip5* in *L. pneumophila* and *L. longbeachae* infected macrophages from C57Bl/6 mice was 5-6.0 fold higher compared to uninfected macrophages. In contrast,

A/J mice macrophages infected by both bacterial species showed ~ 3 fold increase in *naip5* expression compared to uninfected cells. By 12 h post infection, the level of *naip5* expression was down to ~ 3.0 fold increase for infected C57Bl/6 mice macrophages and 2.0 fold increase for A/J mice infected macrophages. When the ratio of *naip5* expression in infected C57Bl/6 macrophages: infected A/J mice macrophages was compared, the result showed that the ratio was 2.8 and 2 fold for *L. pneumophila* and *L. longbeachae* infected C57Bl/6 macrophages, respectively (Fig 15 C). By 6 h post infection of C57Bl/6 macrophages, there ratio was 7.0 and 5 for *L. pneumophila* and *L. longbeachae*, respectively (Fig 15 C). By 12 h post infection of C57Bl/6 macrophages, the expression ratio was 3.1 and 3.9 for *L. pneumophila* and *L. longbeachae* infected C57Bl/6 macrophages, respectively (Fig 15 C). At all the time points there was slightly higher level of C57Bl/6:A/J ratio of *naip5* in *L. pneumophila* infected macrophages compared to *L. longbeachae* infected macrophages ($p < 0.0071-0.014$). This may be due to the induction of slightly higher levels of *naip5* in *L. pneumophila* infected C57Bl/6 macrophages compared to infection by *L. longbeachae*. The above results showed that the susceptible A/J mice macrophages expressed lower level of *naip5* in response to infection by both species of *Legionella* compared to resistant mice macrophages.

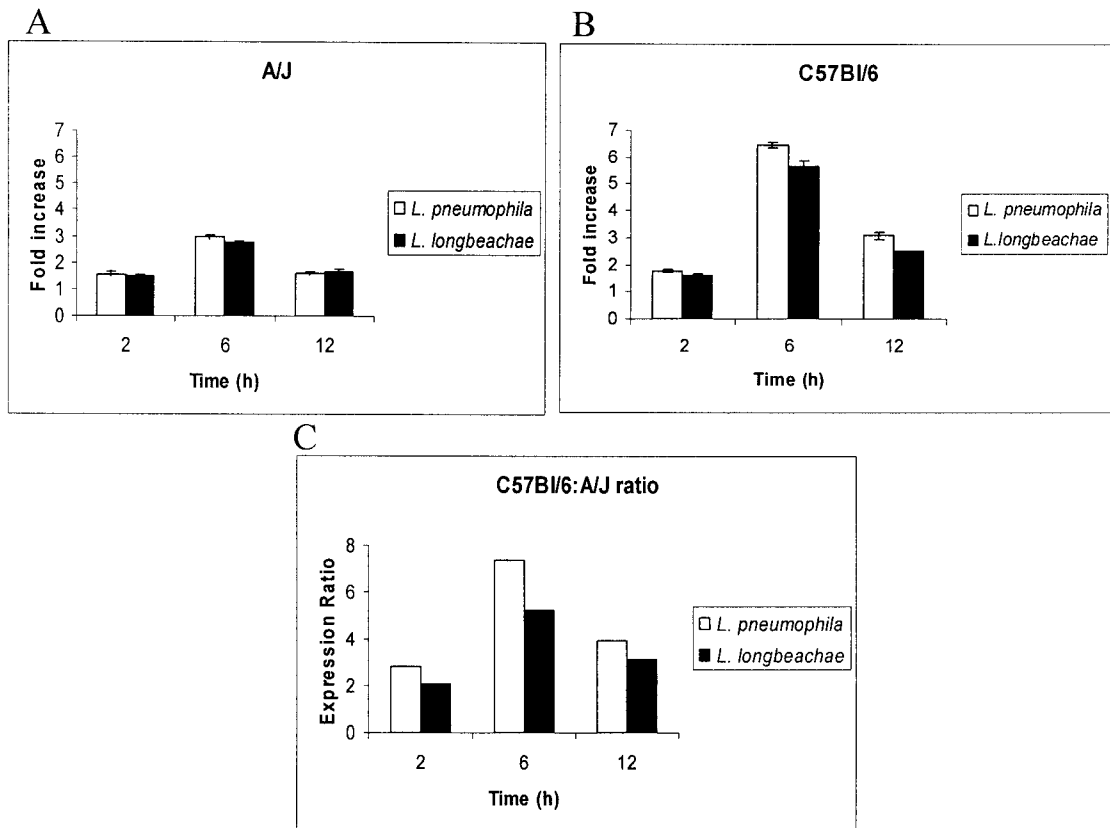


Figure 18. High level of expression of *naip5* in immortalized bone marrow derived macrophages from C57Bl/6 compared to macrophages from A/J mice. Immortalized bone marrow-derived macrophages from A/J and C57Bl/6 mice were infected with *L. longbeachae* strain D 4968 or *L. pneumophila* strain AA100 at MOI of 10 and total RNA was isolated from macrophages at 2, 6 and 12 h post infection. The levels of *naip5* expression in the infected, as well as uninfected cells were determined by real time PCR. mRNA of β -actin was used as the internal control. Levels of expression of *naip5* were determined relative to uninfected cells and are expressed as ratios C57Bl/6 to A/J mice macrophages. The results are representative of two independent experiments, and the error bars represent standard deviations.

***L. longbeachae* induce low level of Caspase-3 activation**

Previous studies have shown that *L. pneumophila* activates caspase-3 by a Dot/Icm-dependent process (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Gao and Abu Kwaik 2000; Zink, Pedersen et al. 2002; Molmeret, Zink et al. 2004). The

activation of caspase-3 and induction of apoptosis is both time- and dose-dependent but is independent of bacterial replication (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Zink, Pedersen et al. 2002; Abu-Zant, Santic et al. 2005). Inhibition of caspase-3 results in fusion of the LCPs to lysosomes (Molmeret, Zink et al. 2004). Some homologues of the IAP family of proteins, of which Naip5 is a member, inhibit caspase-3 and caspase-7 (Deveraux, Takahashi et al. 1997; Roy, Deveraux et al. 1997; Maier, Lahoua et al. 2002). In addition, a murine IAP homologue has also been found to inhibit apoptosis mediated by interleukin 1 β converting enzyme (Uren, Pakusch et al. 1996). Therefore, we determined the ability of *L. longbeachae* to activate caspase-3 and to trigger subsequent apoptosis. We decided to use U937 cells for the caspase-3 and Tunnel assays, since *L. pneumophila*-induced activation of caspase-3 and subsequent apoptosis have been very well established in U937 cells and other human-derived cells (Zink, Pedersen et al. 2002) but very little, if any data, are available in mouse macrophages. We infected U937 macrophages with the 3 clinical isolates of *L. longbeachae* (D 4968, D 4969 and D 4973) at MOI of 50 and determined caspase-3 activity using a caspase-3-specific fluorescent substrate at 6 h post infection. The MOI of 50 was used as previously described (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Zink, Pedersen et al. 2002; Abu-Zant, Santic et al. 2005) to boost and speed up caspase-3 activation to a level detectable by the caspase-3 activity fluorescent assays. Our data showed that compared to the *dotA* mutant negative control, *L. pneumophila* showed very high level of caspase-3 activation ($p < 0.0001$) (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Zink, Pedersen et al. 2002; Abu-Zant, Santic et al. 2005). However, unlike *L. pneumophila*, all three strains of *L. longbeachae* showed much lower level of caspase-3 activation (~60

AFUs) compared to *L. pneumophila* (~180 AFUs) ($p < 0.0001$) (Fig 16 A). To verify that the differences in the level of active caspase-3 is not due to difference in the numbers of bacterial as a result of intracellular replication, we determined growth kinetics of the two bacteria species in U937 cells. As shown in Fig 16 B both bacteria replicated to the same extent in these cells indicating that the differences in caspase-3 activation is due to signals produced by the two bacteria.

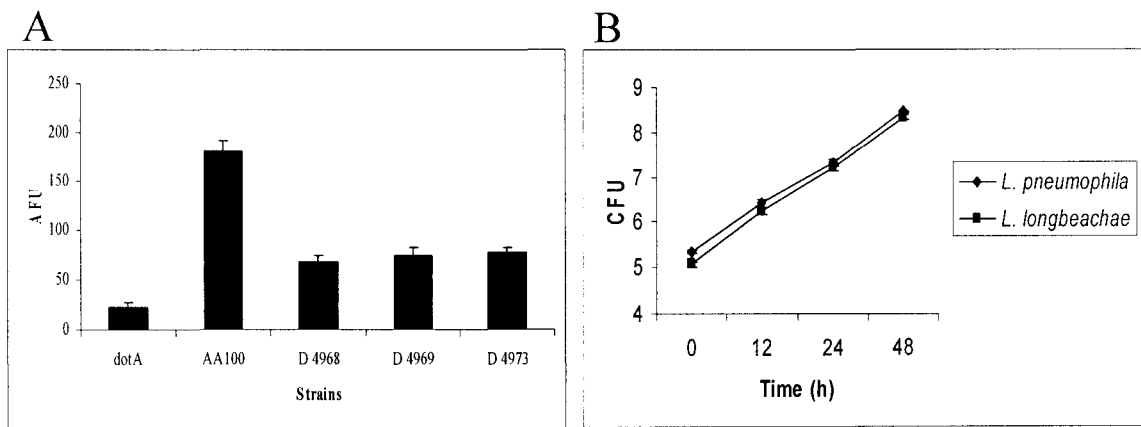


Figure 19. Detection of caspase-3 activation by its cleavage of a fluorescent substrate. U937 macrophages were infected with (A) *L. longbeachae* clinical isolates D 4968, D 4969, D 4973, *L. pneumophila* strain AA100 or its *dotA* isogenic mutant and (B) *L. longbeachae* strain D 4968 and *L. pneumophila* AA100 at MOI of 10. The growth of the two species of *Legionella* in the macrophages was determined and caspase-3 activity, measured by the cleavage of fluorescent substrate, was expressed as the arbitrary fluorescence units. The data are representative of three independent experiments and the error bars represent standard deviations.

To confirm the low level of activation of caspase-3 by *L. longbeachae*, we performed single cell analysis by confocal microscopy to determine the kinetics of caspase-3 activation during the course of infection at several stages of intracellular replication. U937 macrophages were infected with *L. pneumophila* strain AA100 and *L.*

longbeachae clinical isolate D 4968 at MOI of 10 and the activation of caspase-3 was determined by confocal microscopy at 6h and 24h post infection. The *L. pneumophila* AA100 isogenic *dotA* mutant was used as the negative control. At 6h post infection, 50% of *L. pneumophila*-infected macrophages showed caspase-3 activation compared to 16% activation by the *dotA* infected macrophages ($p < 0.0001$). In contrast, the level of caspase-3 activation by *L. longbeachae*-infected macrophages was only 18%, similar to the *dotA* mutant infected macrophages ($p < 0.1$) (Fig 17). By 24h post-infection, more than 85% of *L. pneumophila*-infected macrophages were positive for active caspase-3 compared to 19% for the *dotA* mutant infected macrophages, and 33% of *L. longbeachae*-infected macrophages. The number of infected macrophages that exhibited caspase-3 activation at 24h was slightly higher in *L. longbeachae* infected macrophages compared to cells infected by the *dotA* isogenic mutant of *L. pneumophila* negative control ($p < 0.005$), but much less than *L. pneumophila* Strain AA100 ($p < 0.0007$) (Fig 17). This may be due to proliferation of *L. longbeachae* compared to the *dotA* mutant, which does not replicate. However bacterial numbers alone could not account for the activation of caspase-3, since *L. longbeachae* and *L. pneumophila* replicated to the same level but showed significant difference ($p < 0.0007$) in the level of active caspase-3. The slight increase level of caspase-3 activation at 6 h by *L. longbeachae* strain D 4968 over the *dotA* mutant in the fluorescent caspase-3 activity assay compared to the confocal microscopy may due to higher MOI used in the caspase-3 activity.

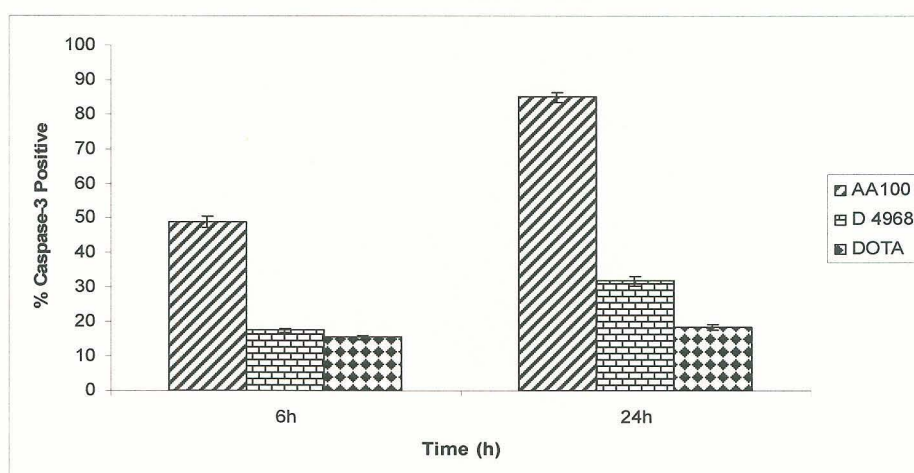
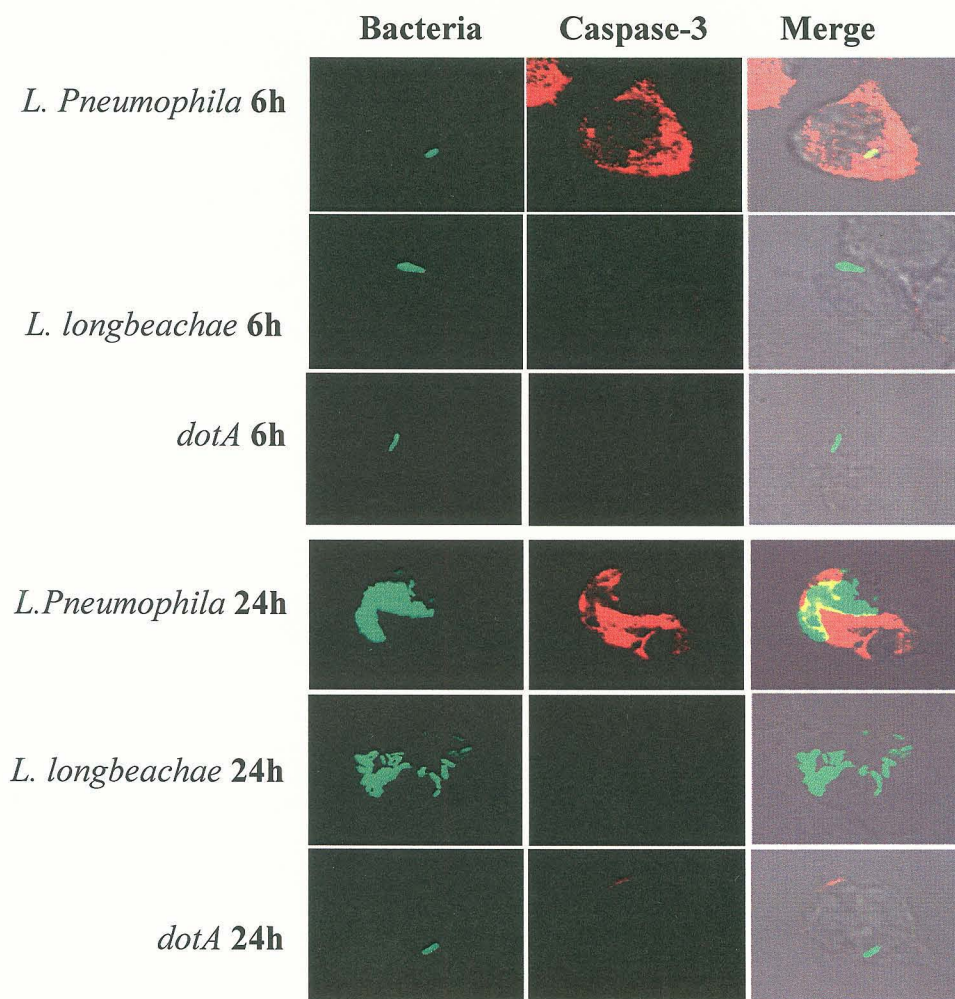


Figure 20. Detection of caspase-3 activation by confocal microscopy. U937 macrophages were infected with *L. longbeachae*, *L. pneumophila* strain AA100 or its *dotA* isogenic mutant at MOI of 10 and caspase-3 activation analyzed at 6 h and 24 h post infection. Bacteria were detected by a specific antibody (green)

while active caspase-3 was detected by an anti-active caspase-3 antibody (red). (A) Representative confocal microscopy images at several time points are shown. (B) Quantification of the % of cells with active caspase-3 was determined by analysis of 100 infected cells from three different coverslips in each experiment. There were equivalent numbers of bacteria per phagosome for both bacteria. The data are representative of three independent experiments and the error bars represent standard deviations. Some of the error bars are too small to be displayed by the software.

***L. longbeachae* induce low level of apoptosis in U937 macrophages**

It has been shown that *L. pneumophila* induces caspase-3 activation during early stages of infection but the caspase-3-mediated apoptosis is triggered during late stages of infection of macrophages (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999; Abu-Zant, Santic et al. 2005). Inhibition of caspase-3 blocks the *L. pneumophila*-induced apoptosis (Gao and Abu Kwaik 1999; Gao and Abu Kwaik 1999). We used TUNEL assay to examine the ability of *L. longbeachae* to induce apoptosis. U937 macrophages were infected by *L. longbeachae* clinical isolate D 4968 or *L. pneumophila* AA100 at MOI of 10 and the induction of apoptosis was determined at 6 h, and 24 h post infection. The *dotA* mutant, which is totally defective in activation of caspase-3 and apoptosis (Zink, Pedersen et al. 2002) was used as a negative control. There was no significant difference in apoptosis upon infection by either species at 6h post-infection compared to the *dotA* mutant. Approximately 5% of all the infected macrophages showed apoptotic nuclei in *L. pneumophila* and *L. longbeachae* as well as the *dotA* mutant infected macrophages, and there was no significant difference between them ($p < 0.10-0.30$). At 24h post-infection, ~ 90% of *L. pneumophila*-infected cells became apoptotic compared to 34% of *L. longbeachae*-infected cells ($p < 0.0005$). Only ~ 11% the cells infected by

the *dotA* mutant of *L. pneumophila*, which was used as the negative control (Zink, Pedersen et al. 2002), became apoptotic by 24h post-infection (Fig 18). This was significantly less than the level of apoptosis induced by *L. longbeachae* clinical isolate D 4968 ($p < 0.02$). The data show that there is a clear correlation between caspase-3 activation and the level of apoptosis in infected cells. We conclude that *L. longbeachae* triggers low level of activation caspase-3 resulting in a low level in the induction of apoptosis at late stages of infection of macrophages.

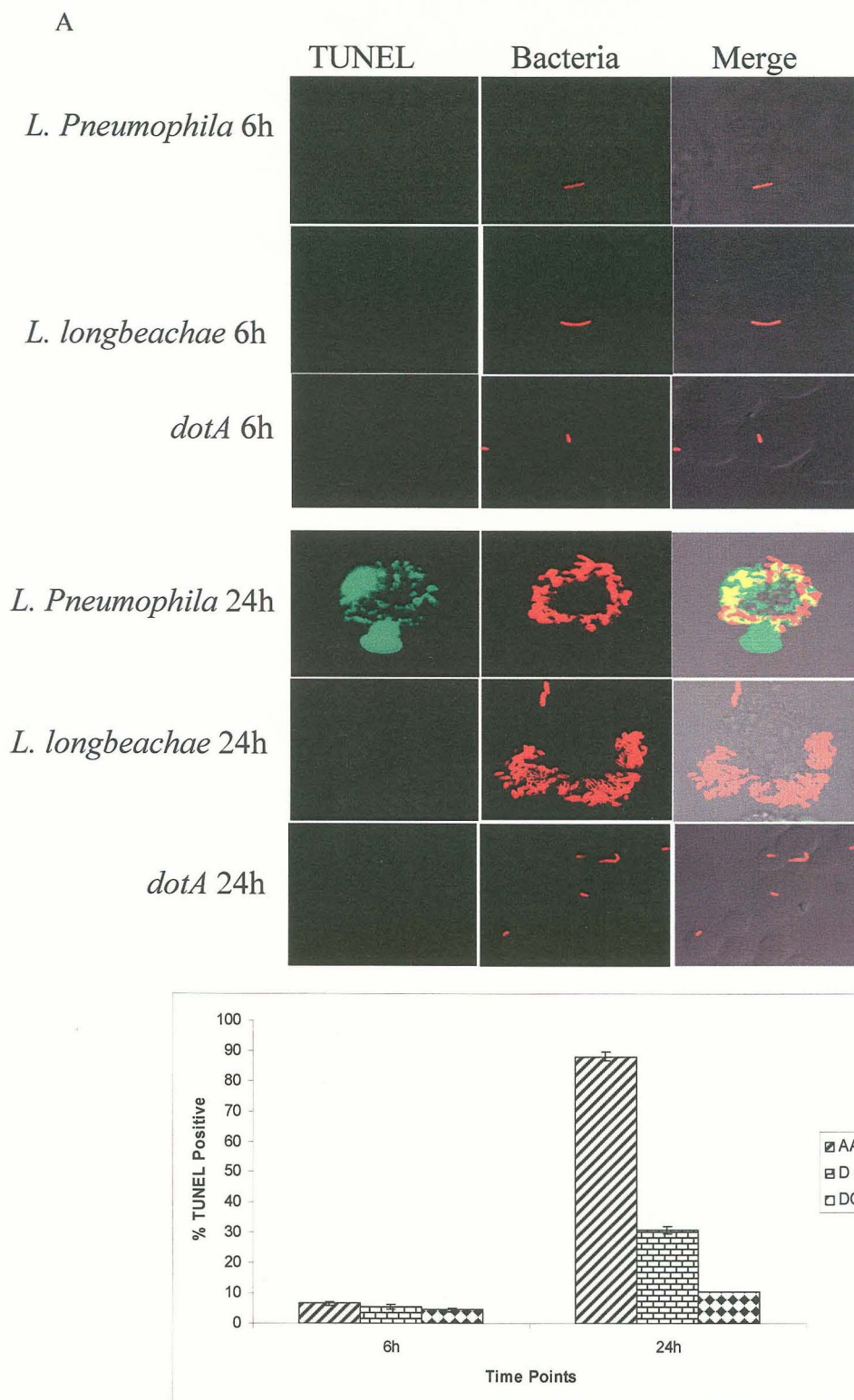


Figure 21. *L. longbeachae* does not induce late stage apoptosis in U937 macrophages. U937 macrophages were infected with *L. longbeachae* strain D 4968, *L. pneumophila* strain AA100 or its *dotA*

isogenic mutant at MOI of 10 and the level of apoptosis in infected cells analyzed at 6 and 24 h post infection. The bacteria were labeled with an antibody (red) while apoptotic nuclei were detected by TUNEL (green). Representative images at the indicated time points are shown in (A). (B) Quantification of the % of apoptotic cells was determined by analysis of 100 infected cells from three different coverslips in each experiment. The data are representative of three independent experiments and the error bars represent standard deviations. Some of the error bars are too small to be displayed by the software.

***L. longbeachae* induce very low pulmonary apoptosis in A/J mice**

Similar to the *in vitro* data in U937 macrophages (Abu-Zant, Santic et al. 2005), we have shown that *L. pneumophila* induces pulmonary apoptosis in A/J mice by 24-48h post infection (Santic' et al, submitted for publication). Our data showed that unlike *L. pneumophila*, *L. longbeachae* induce lower level of apoptosis *in vitro* in U937 macrophages. Therefore, we examined by in situ cell analyses, apoptosis in the lung tissue of A/J mice infected with *L. longbeachae* using laser scanning confocal microscopy. *L. pneumophila* infected mice was used as positive control and uninfected mice was used as a negative control. At different time points after infection, the lungs were embedded in paraffin, cut, fixed, permeabilized, and labeled for apoptosis by TUNEL. Any nuclei stained black by TUNEL were considered apoptotic, regardless of intensity of the staining.

Apoptosis was observed at 24h after infection by *L. pneumophila* strain AA100 in the permissive A/J mice strain, where most of the pulmonary cells underwent apoptosis. Similar to the 24h infection, at 48h and 72h after infection, most pulmonary cells were apoptotic in the lung tissue of A/J mice infected with *L. pneumophila* strain AA100 (Fig. 19). Unlike *L. pneumophila*, there was very low level of apoptosis in the *L. longbeachae*-infected lung tissue up to 7 days post-infection (Fig. 19 and data not shown). Taken together, these results showed that in contrast

to *L. pneumophila*, *L. longbeachae* induce very low level of late stage macrophages apoptosis either *in vitro* or in experimental animals.

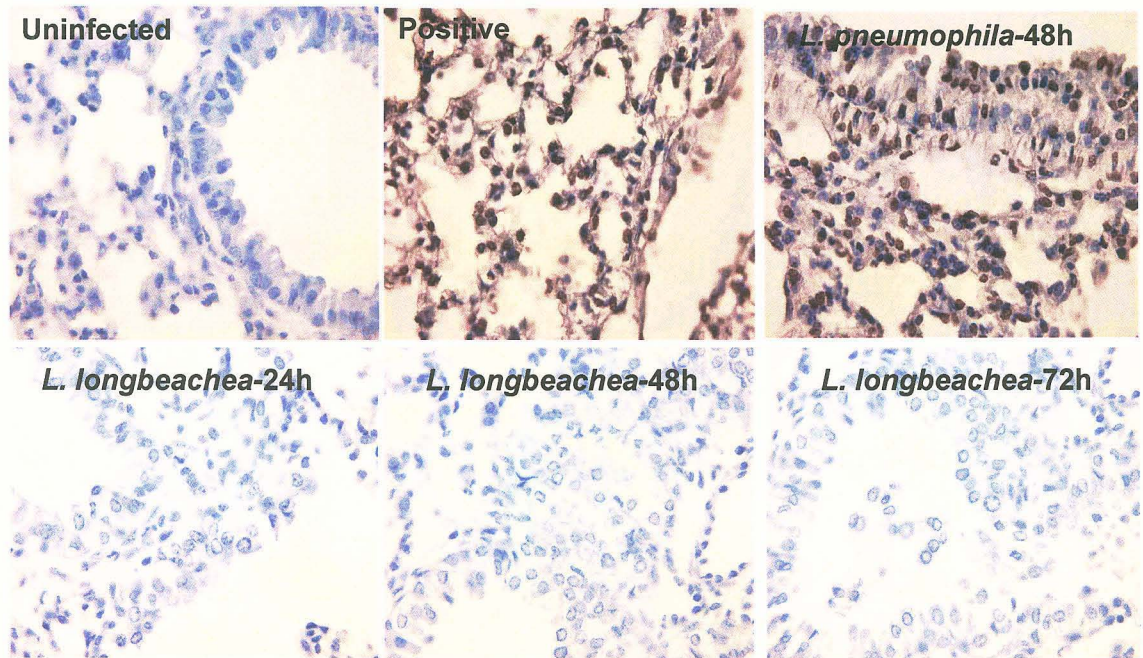


Figure 22. *L. longbeachae* induce low levels of pulmonary apoptosis in A/J mice. A/J mice were infected with 10^6 of *L. longbeachae* strain D 4968 or *L. pneumophila* strain AA100. Lungs from the infected mice were harvested at 24, 48 and 72 h and stained for TUNEL to detect apoptotic nuclei (black). Only 48 h time point is shown for *L. pneumophila* because results from all the time points are similar. The images are representative of 20 different microscopic fields from the lungs of three animals for each time point. The results are representative of three independent experiments.

DISCUSSION

There are several evidences that show that IFN- γ plays an important role in elimination of *L. pneumophila* from the lungs of infected animals. (Horwitz and Silverstein 1981; Bhardwaj, Nash et al. 1986; Blanchard, Friedman et al. 1988; Brieland, Freeman et al. 1994; Heath, Chrisp et al. 1996; Deng, Tateda et al. 2001; Neild and Roy 2004). Whereas quiescent macrophages are susceptible to *L. pneumophila* infection, IFN- γ activated macrophages inhibit the intracellular growth but do not kill the bacterium (Horwitz and Silverstein 1981; Bhardwaj, Nash et al. 1986; Nash, Libby et al. 1988). Moreover IFN- γ treatment of the susceptible A/J macrophages result in inhibition of growth of *L. pneumophila* (Klein, Yamamoto et al. 1991). It has been demonstrated that NK cells are likely to be the first source of IFN- γ produced after infection of the host with *L. pneumophila* (Blanchard, Friedman et al. 1988; Deng, Tateda et al. 2001). Our results show that similar to *L. pneumophila*, IFN- γ inhibits the growth of *L. longbeachea*.

It is interesting that while human monocytes treated with low amounts of IFN- γ restrict the growth of *L. pneumophila*, they are not killed inside these cells. It was determined that the restriction of *L. pneumophila* growth in IFN- γ -activated macrophages is due in part to sequestration of intracellular iron (Byrd and Horwitz, 1989). Within human monocytes, *L. pneumophila* growth is dependent on the influx of iron, a processes mediated by the transferrin receptor (Byrd and Horwitz 1989). When monocytes are activated by IFN- γ , transferrin receptor is downregulated, limiting iron availability and restricting the growth of *L. pneumophila* (Byrd and Horwitz 1989). Interestingly *L.*

pneumophila in activated human monocyte-derived macrophages and A/J mouse bone marrow-derived macrophages fail to alter the biogenesis of its phagosome. The phagosome shows co-localization with LAMP-2, Cathepsin D and Texas red ovalbumin (Santic, Molmeret et al. 2005). Whereas simultaneous pretreatment of macrophages with IFN- γ and iron at 24 h before infection abrogates inhibition *L. pneumophila* replication (Byrd and Horwitz 1989; Santic, Molmeret et al. 2005), iron supplementation at 1 h before infection does not reverse the growth inhibitory effect of IFN- γ (Santic, Molmeret et al. 2005). It is possible that overloading the macrophages with iron over a long time interferes with endocytic trafficking in the IFN- γ activated macrophages. One of the pathways that is upregulated in IFN- γ activated infected macrophages is the production of nitric oxide (NO). Although NO has been found to play an important role in the immune response against *L. pneumophila* in A/J mice, its effect appears to be indirect (Neild and Roy 2004). Thus NO may be involved in the maturation of the LCP to a phagolysosome. However inhibition of *L. pneumophila* replication may be a more direct effect of the IFN- γ activation of macrophages, and it seems likely that *L. longbeachae* growth restriction occurs by similar mechanisms, but further experiments are needed to determine the similarities and differences.

Most bacteria that inhabit disparate environments must alter their physiology to tolerate and adapt to local conditions (Molofsky and Swanson 2004). Some bacteria, especially those that are exposed to harsh environmental conditions during transmission, or in tissues can also respond to environmental cues by activating morphogenetic programs resulting in cellular differentiation (Molofsky and Swanson 2004). Differentiation allows these bacteria to transmit from dying host cells to new hosts.

Notable examples are two obligate intracellular pathogens, *Coxiella burnetii* which alternates between the replicative large cell variant and the environmental small variant (Samuel, Kiss et al. 2003), and *Chlamydia trachomatis*, which changes between an intracellular replicative reticulate body and the resilient and infectious elementary body (Hammerschlag 2002). In natural and potable fresh water supplies, *L. pneumophila* probably resides within biofilm communities, where it falls prey to grazing amoebae. When ingested, the microbe can resist digestion and, instead, replicates profusely before killing its protozoan host and returning to the aquatic reservoir. Although *L. longbeachae* reside in soil it is likely that it is subject to similar condition as it alternates between life within protozoa and biofilms in soil. As predicted for a microbe that transits between phagocytes and water, the *L. pneumophila* life cycle consists of at least two phases (Rowbotham 1986; Garduno, Garduno et al. 2002). *L. pneumophila* has coupled the morphogenetic expression transmissible traits to stationary phase physiology. It is well documented that the ability of *L. pneumophila* to replicate intracellularly is totally dependent on the bacterial growth phase (Byrne and Swanson 1998). While post-exponential *L. pneumophila* can replicate intracellularly, exponentially-grown *L. pneumophila* is completely defective in intracellular replication (Byrne and Swanson 1998; Hammer and Swanson 1999; Bachman and Swanson 2001; Hammer, Tateda et al. 2002). In addition, to its ability to replicate inside cells, *L. pneumophila* at post exponential growth phase in laboratory cultures exhibit other virulence-related phenotypes such as cytotoxicity, stress resistance, motility, sodium chloride sensitivity, and evasion of phagosome-lysosome fusion (Byrne and Swanson 1998; Hammer and Swanson 1999; Bachman and Swanson 2001; Hammer, Tateda et al. 2002). Similarly,

Alli *et al.* have shown that pore formation is growth phase-regulated both *in vitro* and *in vivo* (Alli, Gao et al. 2000). Our studies have shown that the ability of the Philadelphia and AA100 clinical isolate strains of *L. pneumophila* to replicate intracellularly is growth phase-dependent. *In vitro* studies have shown that nutrient limitation at the post-exponential phase is one signal that triggers dramatic phenotypic modulations in *L. pneumophila*, and it is also likely the signal that triggers virulence traits at the post-exponential phase within macrophages (Byrne and Swanson 1998; Hammer and Swanson 1999; Bachman and Swanson 2001; Hammer, Tateda et al. 2002). Expression of the virulence-related phenotypes by *L. pneumophila* at the post-exponential phase is triggered by the stringent response and mediated by ppGpp. This nucleotide accumulates when amino acids become limiting at the post-exponential phase of growth (Hammer and Swanson 1999; Bachman and Swanson 2001). It is believed that ppGpp acts as an alarmone and activates the two-component system regulator, LetA/S, which enables *L. pneumophila* to express its full arsenal of virulence properties within broth-grown cultures (Hammer, Tateda et al. 2002). Our data show that unlike *L. pneumophila*, the ability of *L. longbeachae* to replicate intracellularly is totally independent of the growth phase of the infecting bacteria. In addition, we have shown that *L. longbeachae* is motile at all growth phases, in contrast to *L. pneumophila* that becomes motile only at the post-exponential phase. It is thought that *L. pneumophila* represses its virulence traits that are required for invasion during intracellular replication in the nutrient-rich intracellular environment (Abu Kwaik 1998; Molofsky and Swanson 2004). However, upon depletion of nutrients in spent host cells, the virulence traits are activated to trigger bacterial release to allow secondary infections (Abu Kwaik 1998; Molofsky and Swanson 2004). Since *L.*

longbeachae does not exhibit the growth phase-dependent phenotype, it is likely that it utilizes a different mechanism to adapt to unfavorable conditions but this needs to be examined. This is not surprising since *L. longbeachae* inhabits soil environment in contrast to *L. pneumophila* that inhabits the aquatic environment.

Examination of trafficking of *L. pneumophila* in human macrophages has shown that this bacterium resides in a phagosome that does not interact with the endocytic pathway (Horwitz 1983; Horwitz 1983; Horwitz and Maxfield 1984; Clemens and Horwitz 1995; Clemens, Lee et al. 2000; Clemens, Lee et al. 2000). The *L. pneumophila* phagosome excludes both early and late endosomal markers including Rab5, LAMP-1, LAMP-2 and Cathepsin D (Clemens and Horwitz 1995; Clemens, Lee et al. 2000; Clemens, Lee et al. 2000). The *L. pneumophila* phagosome is only mildly acidified (pH of 6.2-6.3) resembling the pH of an early endosome (Horwitz and Maxfield 1984; Mellman 1996) similar to *Mycobacterium tuberculosis*, but quite distinct from *Salmonella typhimurium*. This isolation of the LCP from the endocytic pathway is dependent on the Dot/Icm secretion system, since mutants of the Dot/Icm secretion system acidify and fail to avoid fusion with the lysosome (Marra, Blander et al. 1992; Berger and Isberg 1993; Sadosky, Wiater et al. 1993; Brand, Sadosky et al. 1994; Vogel, Roy et al. 1996; Segal and Shuman 1997; Andrews, Vogel et al. 1998; Segal, Purcell et al. 1998; Vogel, Andrews et al. 1998). In addition to isolation of the LCP from the endocytic pathway, the Dot/Icm secretion system is involved in recruitment of the ER to the phagosome (Berger and Isberg 1993). There are *L. pneumophila* mutants (Lp120) that reside in phagosomes that are rapidly transported to the lysosome but retain their ability to recruit the ER (Swanson and Isberg 1996). In addition, certain other mutants (Lp112 and Lp172) inhabit

phagosomes that avoid fusion to the lysosomes but are unable to recruit the ER (Swanson and Isberg 1996), suggesting that different proteins injected by the Dot/Icm secretion system mediate these independent processes. It has been shown that *L. longbeachae* possess an intact type IV secretion system (Feldman and Segal 2004). It was shown by Feldman and Vogel that instead of transported effector encoded by *icmQ*, *L. longbeachae* has a *ligA* gene, which can complement loss of *icmQ* from *L. pneumophila* (Feldman and Segal 2004). This indicates that *L. longbeachae* likely translocate similar but not identical substrates, which may perform similar and distinct functions.

Our results showed that in contrast to *L. pneumophila*, the *L. longbeachae* containing phagosome co-localizes with the early endosomal marker EEA1 and the late endosomal markers LAMP-2 and M6PR. The *L. longbeachae* containing phagosome does not acquire the vATPase proton pump, the lysosomal markers Cathepsin D, or the lysosomal tracer, TROV. Interestingly both types of *Legionella* phagosomes recruit the RER. These results show that *L. longbeachae* modulates biogenesis of its phagosome into a late endosome-like stage that does not merge with the lysosomes but acquires ER-like characteristics. Thus the trafficking of *L. longbeachae* resembles trafficking of *Brucella abortus*, which also possesses a functional type IV secretion system related to the Dot/Icm system (Gorvel and Moreno 2002). Although both *L. pneumophila* and *B. abortus* phagosomes interact with the ER there are notable differences in the host pathway that are exploited for the transport of both bacteria to the ER-like compartment. In contrast to *L. pneumophila*, which intercepts the early secretory pathway (Kagan and Roy 2002), *B. abortus* is transported to the ER-like compartment through the autophagy pathway (Swanson and Isberg 1995; Pizarro-Cerda, Meresse et al. 1998; Pizarro-Cerda, Moreno et

al. 1998; Amer and Swanson 2005). Unlike *L. pneumophila*, early phagosome containing *Brucella* engage the early endocytic pathway and are acidified shortly after formation (Pizarro-Cerda, Meresse et al. 1998). In macrophages, the *Brucella*-containing vacuole (BCV) acquires the early endosomal marker EEA1 and the late endosomal marker LAMP-1 early during infection (Celli, de Chastellier et al. 2003). Similar to the BCV, *L. longbeachae* phagosome co-localizes with early and late endosomal markers but does not mature to a phagolysosome stage. Similar to *L. pneumophila* and *B. abortus*, *L. longbeachae* is targeted to the ER. However unlike the BCV, which begins to lose LAMP-1 by 4 h post-infection (Celli, de Chastellier et al. 2003), the *L. longbeachae* containing phagosome maintains LAMP-2 up to 12 h post-infection. Moreover the *L. longbeachae* phagosome co-localizes with M6PR in contrast to the BCV. Our data indicate that trafficking of *L. longbeachae* bears more resemblance to *B. abortus* than *L. pneumophila* and its trafficking is important and so far seems to be unique compared to other intracellular pathogens, and clearly different from that of *L. pneumophila*.

Trafficking of *L. longbeachae*, similar to the BCV, resembles biogenesis of an autophagosome (Swanson and Isberg 1995; Pizarro-Cerda, Meresse et al. 1998; Pizarro-Cerda, Moreno et al. 1998; Amer and Swanson 2005). Autophagy is a mechanism by which eukaryotic cells capture unwanted cytoplasmic components and damaged organelles for delivery to the lysosomes (Dunn 1990; Seglen, Gordon et al. 1990). A hallmark of autophagy is the sequestration of cytoplasmic components into a double-membrane vacuole known as the autophagosome (Dunn 1990; Seglen and Bohley 1992). As the autophagosome matures, it acquires late endosomal/lysosomal markers such as LAMP-2 (Dunn 1990; Kopitz, Kisen et al. 1990). Similar to autophagosome, the *L.*

longbeachae-containing phagosome is surrounded by the ER and acquires LAMP-2 and the M6PR markers. Whether *L. longbeachae* modulates autophagy is still to be determined.

Despite the differences in intracellular trafficking between the two *Legionella* species, *L. longbeachae* can replicate in a communal phagosome with *L. pneumophila*. This is further confirmed by the ability of *L. longbeachae* to rescue the isogenic *dotA* mutant of *L. pneumophila* for intracellular replication in a communal phagosome that is expected to resemble that of *L. longbeachae*. It is more likely that biogenesis of the communal phagosomes harboring *L. longbeachae* and the *dotA* mutant of *L. pneumophila* is remodeled by *L. longbeachae*, since the *dotA* mutant is completely defective in export of Dot/Icm effectors required for early arrest of phagosome biogenesis. Therefore, *L. pneumophila* is capable of replicating within the *L. longbeachae*-containing phagosome that has late endosome-like characteristics. Additional experiments would determine whether *L. longbeachae* is also capable of replicating in a phagosome remodeled by *L. pneumophila* by constructing *dot/icm* mutants of *L. longbeachae* and testing the ability to replicate within phagosomes remodeled by *L. pneumophila*.

In human macrophages the LCP excludes all endosomal and lysosomal markers throughout the intracellular infection (Clemens and Horwitz 1995; Clemens, Lee et al. 2000; Clemens, Lee et al. 2000). In contrast during late stages of infection of murine macrophages, *L. pneumophila* replicates in LAMP and Cathepsin D-positive, acidified phagosomes (Sturgill-Koszycki and Swanson 2000). It is possible that the delay in maturation of the LCP in murine macrophages may be necessary for *L. pneumophila* to acquire the right phenotype to enable it to replicate during late stages in mature acidic

phagolysosomal compartment. It may not be surprising then that *L. pneumophila* replicates in a communal phagosome established by *L. longbeachae*, which is a late endosome-like compartment. It is also possible that maturation of the *L. longbeachae* phagosome to a late endosome-like compartment is not a prerequisite for bacterial replication. In contrast to our results, Sauer et al previously showed that *L. pneumophila* could not replicate in communal phagosome with *C. burnetii* (Sauer, Shannon et al. 2005). However there is a difference between *L. longbeachae* phagosome and that of *C. burnetii*. Unlike the *L. longbeachae* phagosome, which does not co-localize with vATPase, the *C. burnetii* phagosome becomes acidified.

In addition to the differences in endocytic trafficking, a major difference between *L. pneumophila* and *L. longbeachae* is genetic susceptibility of inbred mice strains to infection. Previous studies have shown that all inbred mice strains including C57Bl/6, Balb/c, CH3/HeN, BDF1 and DBA/2 are resistant to *L. pneumophila* infection with the exception of A/J mice (Hedlund, McGann et al. 1979; Yoshida and Mizuguchi 1986; Yamamoto, Klein et al. 1987; Yamamoto, Klein et al. 1988; Yamamoto, Klein et al. 1988; Yoshida, Goto et al. 1991; Miyamoto, Maruta et al. 1996; Izu, Yoshida et al. 1999). Most other *Legionella* species can however establish infection in these mice (Miyamoto, Maruta et al. 1996; Izu, Yoshida et al. 1999). The differences in genetic susceptibility have been mapped to a polymorphism in the *naip5* allele of chromosome 13 (Yoshida, Goto et al. 1991; Beckers, Yoshida et al. 1995; Dietrich, Damron et al. 1995; Beckers, Ernst et al. 1997; Diez, Lee et al. 2003; Wright, Goodart et al. 2003). The genetic basis for the inhibition of replication by polymorphism in the *naip5* allele is not understood. In this report, we have shown efficient intrapulmonary replication of *L. longbeachae* in A/J,

C57Bl/6 and Balb/c mice. Our results show that susceptibility of mice to infection by *L. longbeachae* is independent of polymorphism in the *naip5* allele.

It has been shown that when expression of the *naip* family of genes (~8) is examined as a group, their collective expression is induced after phagocytosis of *L. pneumophila*, *Salmonella typhimurium*, or latex beads (Diez, Yaraghi et al. 2000), but whether *naip5* is one of the induced *naip* genes is not known. In addition, A/J mice macrophages express less Naip proteins than C57Bl/6 mice, but whether Naip5 is partially or exclusively responsible for the different level of Naip proteins is not known (Diez, Yaraghi et al. 2000). Our current studies are focused exclusively on expression of the *naip5* gene, since it is the only gene in the *naip* family of genes that determines susceptibility to *L. pneumophila*. Our analyses show that uninfected A/J and C57Bl/6 mice macrophages express *naip5*. Upon infection with *L. pneumophila* and *L. longbeachae*, there is an increase in the level of *naip5* expression. However, the increase in *naip5* transcription was much higher for C57Bl/6 compared to A/J mice macrophages. The expression level peaked around 6h post-infection for both *Legionella* species. However, *L. pneumophila*-infected C57Bl/6 macrophages expressed a slightly higher level of *naip5* compared to *L. longbeachae*-infected C57Bl/6 macrophages. Since *naip5* expression in infected macrophages is induced during early stages of infection, the failure of *L. pneumophila* to replicate within non permissive murine macrophages is correlated with up-regulation of transcription of *naip5*. The level of expression of Naip5 peaks at 6h post infection, which is the time point at which caspase-3 activation is detectable in *L. pneumophila* infected macrophages. Thus, the higher level of expression of Naip5 coincides with activation of caspase-3. Since Naip/Birc1 have been shown to inhibit

caspases 3, 7 and 9 (Deveraux, Takahashi et al. 1997; Roy, Deveraux et al. 1997; Maier, Lahoua et al. 2002; Davoodi, Lin et al. 2004; Fortier, Diez et al. 2005), we speculate that the differential susceptibility of mice strains to *L. pneumophila* may be related to the function of Naip5 as an inhibitor of caspase-3 and as an anti-apoptotic protein. However, whether Naip5 inhibits Caspase-3 is still to be determined. Recently, Naip proteins have been classified in the NACHT-LRR (NLR) family of proteins, which are cytoplasmic proteins involved in intracellular recognition of conserved microbial products (Chamaillard, Girardin et al. 2003; Fortier, Diez et al. 2005). Members of this family of proteins which include NODs, IPAFs and NALPs possess domains such as caspase-recruitment domain (CARD), BIR domains, and pyrin domain (PYD) respectively (Chamaillard, Girardin et al. 2003; Fortier, Diez et al. 2005). These domains are involved in the regulation of pro-apoptotic and pro-inflammatory signaling. IPAF and NALP subfamilies have been shown to activate the pro-inflammatory caspases including caspase-1 and caspase-5 to form inflammasome (Chamaillard, Girardin et al. 2003; Martinon and Tschopp 2004; Fortier, Diez et al. 2005). Whether Naip5 is involved in inhibition of pro-inflammatory caspases, such as caspase-1, is also still to be determined.

L. pneumophila activates caspase-3 early during infection in a Dot/Icm-dependent process (Gao and Abu Kwaik 1999). The activation of caspase-3 is essential for the isolation of the LCP from the endocytic pathway, since inhibition of caspase-3 results in fusion of the LCP to lysosomes (Molmeret, Zink et al. 2004). Active Caspase-3 cleaves the Rab-5 effector Rabaptin-5 (Cosulich, Horiuchi et al. 1997). Unlike *L. pneumophila*, *L. longbeachae* activates very low level of caspase-3 and low level of apoptosis. The level of caspase-3 activation is similar to what was shown by Arakaki et al

(Arakaki, Higa et al. 2002) We have shown that the *L. longbeachae* phagosome matures to a late endosome-like stage, which is correlated with the very low activation of caspase-3 by *L. longbeachae*. Although caspase-3 is activated early during infection of macrophages by *L. pneumophila*, apoptosis is not triggered until ~18h post-infection (Abu-Zant, Santic et al. 2005). The delayed apoptosis by *L. pneumophila* is similar in both U937 cells, J774 cells (Abu-Zant, Santic et al. 2005) as well as in the lungs of infected A/J mice. Naip proteins activate JNK, which triggers pro-apoptotic signaling (Chen, Wang et al. 1996; Tournier, Hess et al. 2000). Therefore it is possible that the differential susceptibility of mice strains to *L. pneumophila* is a result of the ability of the resistant mice to induce early apoptosis in the presence of caspase-3 activation leading to early macrophage cell death. In this regard, it has been shown that premature cell death occurs in C57Bl/6 macrophages under conditions of high bacterial load, when compared to A/J mice macrophages (Derre and Isberg 2004). On the other hand, Naip5 may function as an inflammasome similarly to NODS and IPAFs. In this regard, low level of Naip5 proteins in A/J mice macrophages could impair its function in inflammatory signaling resulting in the susceptibility of A/J mice macrophages to *L. pneumophila* infection.

Apoptosis plays an important role in modulating the pathogenesis of a variety of infectious diseases (Thompson 1995; Weinrauch and Zychlinsky 1999). A number of studies show that apoptotic death of the host cell may not necessarily be a quiescent one, but rather may contribute to an antimicrobial immune response. For example, mature interleukin-1 β (IL-1 β) is released by *Salmonella* and *Shigella*-infected macrophages undergoing apoptosis leading to the induction of an acute inflammatory response that

eventually clears the infection (Hilbi, Chen et al. 1997; Zychlinsky and Sansonetti 1997; Hersh, Monack et al. 1999). Similarly, *Mycobacterium*-infected macrophages undergoing apoptosis are scavenged by neighboring macrophages, which reduces the viability of the intracellular mycobacterium (Fratazzi, Arbeit et al. 1997). Scavenging of *Salmonella*-infected apoptotic macrophages by dendritic cells results in processing and presentation of bacterial antigens (Yrlid and Wick 2000). These results indicate that apoptosis may be a host cell response to restrict the growth of intracellular bacteria. Unlike the above scenario, induction of apoptosis by *L. pneumophila* is likely to be beneficial to the bacteria. This is supported by the fact that *L. pneumophila* does not induce apoptosis until late stages of infection, when all the nutrients in the host cells have been exhausted and the bacteria would benefit from killing and lysis of the host cell to allow re-infection (Abu-Zant, Santic et al. 2005). Moreover, induction of apoptosis by both the intrinsic and extrinsic pathway at early stages of infection inhibit *L. pneumophila* replication (Abu-Zant, Santic et al. 2005). It is postulated that apoptosis may facilitate *L. pneumophila* to exit the spent host. In terms of modulation of apoptosis, which may be beneficial to the pathogen, *L. pneumophila* may not be unique. Two *Yersinia* translocated proteins, YopJ or YopP binds to and inhibit the activity of the mitogen-activated protein kinase (Orth, Palmer et al. 1999) that results in blocking the activation of NF- κ B (Schesser, Spiik et al. 1998). *Mycobacterium tuberculosis* promotes secretion of soluble tumor necrosis factor (TNF) receptor 2 (sTNFR2) by infected macrophages that neutralizes the pro-apoptotic activity of TNF- α (Balcewicz-Sablinska, Keane et al. 1998; Kornfeld, Mancino et al. 1999). Additionally, the expression of the anti-apoptotic protein Bcl-2 is down regulated in *Mycobacterium*-infected macrophages (Klingler, Tchou-Wong et al. 1997) with

concomitant increase in the production of TNF- α , which promotes apoptosis in a caspase-1-dependent pathway (Keane, Balcewicz-Sablinska et al. 1997; Rojas, Olivier et al. 1999). The importance of these bacterial induced apoptosis to the intracellular survival of these organisms is yet to be determined. It is most likely that *L. pneumophila* induction of apoptosis results in termination of intracellular replication in vivo and may facilitate release of intracellular bacteria. However, apoptotic infected cells may be recognized by macrophages that engulf them, thus limiting bacteria spread.

In contrast to intracellular bacteria, many extracellular pathogens induce the apoptotic pathways indirectly by translocating bacterial toxins onto the host cell plasma membrane or into the host cell cytoplasm. For example, insertion of secreted Neisserial porin (PorB) into the plasma membrane causes influx of extracellular Ca²⁺ and subsequent activation of Ca²⁺-dependent proteases with pro-apoptotic activity (Meyer 1999; Muller, Gunther et al. 1999). Similarly, secreted staphylococcal α -toxin induces apoptosis by inserting into and forming pores in the host cell membranes (Bayles, Wesson et al. 1998). It is possible that most bacterial toxins that induce physical damage to the host cell will trigger the host cell's apoptosis in response to the non-specific physical damage. The significance of apoptosis induced by most of these organisms to survival of the bacteria is not known. Unlike extracellular bacteria, apoptosis induced by *L. pneumophila* is tightly controlled. Although caspase-3 is activated at early stages of infection, apoptosis is not induced until very late infection. Our results show that unlike *L. pneumophila*, *L. longbeachae* induces only a modest level of apoptosis in macrophages during late stage of infection. Similarly, the lungs of A/J mice infected with *L. longbeachae* exhibit a very low detectable level of apoptosis up to 72h post infection.

The failure of *L. longbeachae* to induce significant level of apoptosis indicates that the bacteria may use a different mechanism to kill the host cell upon termination of intracellular replication.

In addition to caspase-3 mediated apoptosis which occurs at late stages of infection of mammalian cells (Abu-Zant, Santic et al. 2005), there is also a necrotic cell death that is mediated by the *L. pneumophila* pore-forming activity (Alli, Gao et al. 2000). These two mechanisms may act synergistically to mediate the egress of *L. pneumophila* from the spent host cell. Analysis of integrity of the *L. longbeachae* phagosome at the ultrastructural level reveals that similar to *L. pneumophila*, the *L. longbeachae* phagosome becomes disrupted and the bacteria egress into the cytoplasm during late stages of infection, prior to lysis of the host cell. Previous studies from our laboratory have shown that release of intracellular bacteria (rib) mutant that is defective in the pore-forming activity is defective in egress into the cytoplasm (Gao and Abu Kwaik 2000). However, in contrast to *L. pneumophila*, *L. longbeachae* did not show any detectable contact-dependent hemolytic activity. We speculate that the two bacteria may utilize different substrates to disrupt the phagosomal membrane in order to egress into the cytoplasm. It is also possible that the pore-forming activity of *L. longbeachae* is not detectable by contact-dependent hemolysis of sRBCs.

Conclusions

I conclude that there are dramatic differences and divergence in the virulence mechanisms of *L. longbeachae* and *L. pneumophila*: The ability of *L. longbeachae* to replicate intracellularly is independent of the growth phase of the bacterial inoculum, and the *L. longbeachae* phagosome is trafficked into a late endosome-like stage phagosome that does not merge with the lysosomes. This is a unique trafficking compared to other intracellular pathogens. Based on the electron microscopy, hemolytic assay, and TUNEL assay experiments, I conclude that *L. longbeachae* does not possess contact dependent hemolytic activity nor does it induce apoptosis at late stages of infection indicating that it uses a different mechanism to exit the host cell at the end of intracellular replication in contrast to *L. pneumophila*. I concluded from the caspase-3 activation experiments that *L. longbeachae* does not require caspase-3 activation for its intracellular replication. Moreover, both *L. longbeachae* and *L. pneumophila* induce relatively high level of naip5 expression in C57Bl/6 mice compared to A/J mice. From this I concluded that the inability of *L. pneumophila* to replicate in C57Bl/6 in contrast to A/J mice may be related to function of Naip5 as an inhibitor of caspase-3. Since *L. longbeachae* activates low level of caspase-3, which is not required for intracellular replication, it is able to replicate in C57Bl/6 mice.

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PUBLICATIONS

Abstracts

1. Rexford Asare, Jill Suttles, and Yousef Abu Kwaik, Distinct Intracellular Trafficking of *Legionella longbeachae* and *Legionella pneumophila* 37th Annual General Meeting, SLB Oct. 2004

Meetings

2. Rexford Asare, Marina Santic, and Yousef Abu Kwaik, Genetic susceptibility to *Legionella longbeachae* is independent of allelic polymorphism of *naip5* and intracellular trafficking of the organism is distinct from *Legionella pneumophila*, 11th Annual Midwest Microbial Pathogenesis Conference Oct. 2004
3. R. Asare and Y. Abu Kwaik, Intracellular Trafficking of *Legionella Longbeachae*, 104th ASM General Meeting, May 2004

Manuscripts

1. Rexford Asare, Ivana Gobin, Marina Santic, Miljenko Doric, and Yousef Abu Kwaik, Divergence in the intracellular life style of *Legionella longbeachae* from *Legionella pneumophila* (Submitted for Publication)
2. Rexford Asare, Ivana Gobin, Marina Santic, Miljenko Doric, Jill Suttles, James Graham¹, Chris Price, and Yousef Abu Kwaik, The ability of *Legionella longbeachae* to cause infection in mice is independent of allelic polymorphism of *naip5* (Submitted for publication)
3. Marina Santic, Alaeddin Abu-Zant, Maelle Molmeret, Rexford Asare Andrea Dekanic, Miljenko Doric, and Yousef Abu Kwaik, Dot/Icm-mediated pulmonary apoptosis during experimental Legionnaires' disease in susceptible A/J mice (Submitted for Publication)
4. Molmeret M, Zink SD, Han L, Abu-Zant A, Asari R, Bitar DM, Abu Kwaik Y. Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the Legionella-containing phagosome. Cell Microbiol. 2004 Jan;6(1):33-48.
5. Alaeddin Abu-Zant, Rexford Asare, James E. Graham, Yousef Abu Kwaik. A role for Legionella pneumophila RpoS but not RelA in adaptation to the phagosomal microenvironment and for mation of a replicative niche (in publishing).

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